

STUDIES ON THE IMPACT OF AMINE-CONTAINING COMPOUNDS ON
LYSOSOMES

By

Ryan Sol Funk

Pharm.D., University of Kansas, 2007

M.S., University of Kansas, 2010

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Chairperson Jeffrey P. Krise

Valentino J. Stella

Teruna J. Siahaan

M. Laird Forrest

Rick T. Dobrowsky

Date Defended: September 15, 2011

The Dissertation Committee for Ryan Sol Funk
certifies that this is the approved version of the following dissertation:

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Lysosomes accumulate weakly basic amine-containing drugs (i.e., lysosomotropic amines) through an ion trapping-type mechanism and are capable of disrupting lysosomal structure and function. A common finding following treatment with hydrophobic amine-containing drugs is a cellular lipidosis marked by the lysosomal accumulation of lipids. The impact of this drug-induced lipidosis on the lysosomal accumulation of weakly basic amine-containing drugs is unknown. In this work, hydrophobic ($\log P > 2$) amine-containing drugs (imipramine, haloperidol, risperidone, chlorpromazine, lidocaine, bupivacaine, amiodarone, verapamil, propranolol) were found to cause a significant increase in the cellular accumulation of the lysosomotropic amine, LysoTracker Red (LTR).

Imipramine was further found to specifically increase the cellular accumulation of amine-containing substrates of lysosomal ion trapping (LysoTracker Green, daunorubicin, methylamine, propranolol) but not compounds that don't accumulate by ion trapping in lysosomes, suggesting that the drug-induced lipidosis specifically increases the cellular accumulation of drugs that accumulate in lysosomes. Imipramine-induced accumulation of LTR occurred in a time- and temperature-dependent manner in agreement with an energy-dependent intracellular remodeling process that has been reported to coincide with induction of the cellular lipidosis. Prevention of the

imipramine-induced lipidosis by treatment with 0.1% hydroxypropyl- β -cyclodextrin or growth in lipoprotein-depleted media prevented the increased accumulation of LTR, suggesting the hyperaccumulation of LTR is secondary to the drug-induced lipidosis. Determination of lysosome specific accumulation of LTR in cells with or without an intact lysosomal pH gradient revealed that imipramine caused an approximately three-fold increase in the lysosome-specific uptake of LTR.

Previous reports have suggested that drugs accumulate following a cellular lipidosis through enhanced hydrophobic binding sites. However, these results are consistent with an increased aqueous volume of lysosomes resulting in increased drug accumulation. Theoretical calculations of lysosomal volume changes following imipramine treatment reveal an approximately 4-fold increase in apparent lysosomal volume. Therefore, drug-induced lipidosis is found to increase the cellular accumulation of amine-containing drugs by causing a marked increase in the lysosomal volume of cells. These findings have implications towards understanding how drugs can interact at the intracellular level. The ability of one drug to affect the distribution properties of a second is further hypothesized to result in drug-drug interactions.

This dissertation is dedicated to my wife, Lindsey

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As a pharmacy student at the University of Kansas I realized early on that my interests lie in the scientific underpinnings of drug discovery and development and not necessarily in the practice of pharmacy, for that I thank the outstanding faculty within the University of Kansas School of Pharmacy that helped me come to this realization. It was under the direction of the new dean, Dr. Ken Audus, that I approached Dr. Jeff Krise about working as an undergraduate research assistant. Upon meeting Dr. Krise, I immediately sensed his enthusiasm and commitment as a researcher and as a mentor. Working under Dr. Krise and his first graduate student, Dr. Murali Duvvuri, I discovered my passion for research, for which I owe both of them a debt of gratitude. It was those early successes as an undergraduate researcher that truly motivated me to pursue the Ph.D. degree. As a graduate student, Dr. Krise has given me the scientific freedom to pursue my own project, but most importantly has known when to step in and give me the guidance necessary to make the project a success. Therefore, I would like to thank Dr. Jeff Krise and I hope that someday I can motivate and guide students as he has done for me.

I would like to extend my gratitude to the members of my dissertation committee, Dr. Valentino J. Stella, Dr. Teruna J. Siahaan, Dr. M. Laird Forrest and Dr. Rick T. Dobrowsky. In particular, I would like to thank Drs. Stella and Siahaan for volunteering to read the advanced version of my dissertation and for providing helpful suggestions and comments that have undoubtedly improved my writing skills.

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Chapter 1

Introduction

1.1. Statement of the problem

The ability of drugs that induce the lysosomal accumulation of lipids to influence the cellular accumulation of weakly basic amine-containing drugs and the mechanisms involved are the focus of this work. Weakly basic amine-containing drugs accumulate in lysosomes through an ion trapping-type mechanism that is important in the cellular (Ishizaki, Yokogawa et al. 1996) and tissue (Daniel, Bickel et al. 1995) uptake of these drugs. By accumulating in lysosomes these drugs are capable of altering the structural and functional properties of lysosomes resulting in effects such as: alkalinization of lysosomes (Ohkuma and Poole 1978), accumulation of large cytoplasmic vacuoles (Ohkuma and Poole 1981) and lysosomal accumulation of lipids (Hein, Lullmann-Rauch et al. 1990). These alterations may significantly influence the capacity of lysosomes to participate in the normal accumulation and distribution of amine-containing drugs. Specifically, alkalinization of lysosomes has been found to reduce the cellular and tissue accumulation of amine-containing drugs (Daniel and Wojcikowski 1999). To date no studies have evaluated the effect of drug-induced lysosomal lipid accumulation on the cellular accumulation of amine-containing drugs. Studies into the drug-induced lipidosis have suggested a time-dependent remodeling process resulting in the accumulation of lipid-laden lysosomes (Reasor, Ogle et al. 1988) independent of effects on lysosome pH (Lange, Ye et al. 1998). Such changes are hypothesized to result in the increased cellular accumulation of amine-containing drugs into lysosomes, either by an increase in the volume of the lysosomal compartment or by an increase in intralysosomal binding sites (i.e., lipids) for many of these drugs. The ability of one drug to alter the cellular

accumulation of a secondarily administered drug is further hypothesized to represent a novel pathway for distribution-based drug-drug interactions.

1.2. Objectives of this work

This work seeks to understand the effect of drug-induced lysosomal lipidosis on the cellular disposition of amine-containing drugs and the mechanism involved. Initially, the impact of the drug-induced lipidosis on the cellular accumulation of amine-containing substrates for lysosomal ion trapping will be assessed. The drug-induced lipidosis will be evaluated for its specificity in increasing the cellular accumulation of substrates for ion trapping in lysosomes using a series of substrate and non-substrate molecules. The mechanism of enhanced lysosomal accumulation of these amine-containing drugs will be evaluated using a series of assays to differentiate possible mechanisms of increased retention. Measurements of lysosomal pH, drug uptake and efflux, intralysosomal drug-binding and lysosomal volume will elucidate how the drug-induced lipidosis affects the lysosome-specific retention of these drugs. An understanding of the mechanism by which the drug-induced lipidosis increases the cellular retention of these drugs would allow for the prediction of drugs that would be substrates for this potential drug-drug interaction. The drug-induced lipidosis is found to result in a lysosomal volume expansion that is further tested on its ability to non-specifically inhibit lysosomal enzyme activity as a potential mechanism of drug-induced defects in lysosomal lipid metabolism. Finally, amine-containing drugs are characterized based on their ability to differentially affect lysosomal properties that are important in the cellular accumulation of substrates for lysosomal ion trapping, such as

lysosomal volume and lysosomal pH. Expansion of the lysosomal compartment in the form of a cytoplasmic vacuolization is assessed by light microscopy and the lysosomal lipidosis is measured using a fluorescent phospholipid analog. Lysosomal alkalization is determined using indirect measurements of lysosome pH. The culmination of these effects is hypothesized to impact the lysosomal ion trapping capacity of cells.

1.3. Introduction

A common feature among the majority of currently marketed low-molecular weight drugs is the presence of a weakly basic amine functional group. The presence of this functional group often results in the distribution to, and accumulation in, lysosomes (MacIntyre and Cutler 1988). Lysosomes accumulate these drugs to a high degree because of the low luminal pH of the organelle relative to the rest of the cell (de Duve, de Barsey et al. 1974). Because of the intense accumulation in the lysosome of these amine-containing drugs, these compounds are often referred to as 'lysosomotropic amines'. Drug accumulation in lysosomes is known to impact the pharmacokinetic properties of the drug by providing a tissue site for drug accumulation (Siebert, Hung et al. 2004), often resulting in a large apparent volume of distribution (Yokogawa, Ishizaki et al. 2002). In addition, the accumulation of these drugs in the lysosome has been observed to influence the normal physiological and morphological characteristics of the lysosome itself. These off-target drug effects are gaining interest from drug discovery and development scientists as they may relate to the pharmacological and toxicological effects elicited by many of these drugs. Also, the potential that these off-target effects can impact the ability of lysosomes to contribute to

the tissue uptake and accumulation of drugs reveals the possibility that one drug, by affecting lysosomes, can alter the pharmacokinetic properties of a second resulting in drug-drug interactions.

In this chapter the major concepts and literature related to amine-containing drug accumulation in lysosomes and its importance in drug discovery and development will be reviewed with an emphasis on how drug accumulation in the lysosome impacts organelle structure and function. First, an overview of the theoretical aspects of lysosomal ion trapping of drugs will be covered (Section 1.4). Next, an overview of the literature on the influence of lysosomal accumulation of amine-containing drugs on drug activity and pharmacokinetics will be covered (Section 1.5). Finally, is a discussion on published reports illustrating the ability of amine-containing drugs to alter the normal physiology and morphology of lysosomes resulting in a variety of off-target effects mediated by these drugs (Section 1.6). Specifically, a focused review on the ability of lysosomotropic amines to cause alkalinization of the lysosome, the formation of large cytoplasmic vacuoles and the lysosomal accumulation of lipids will be discussed along with references to their potential pharmacological and toxicological implications. In addition, commentary on the observed relationships between drug hydrophobicity and these different effects will be presented.

1.4. Overview of lysosomal ion trapping

1.4.1. Ion trapping mechanism

The mechanism by which weakly basic amine-containing drugs accumulate in lysosomes is through an ion trapping-type mechanism (de Duve, de Barse et al. 1974).

Like other drug disposition mechanisms the properties of the compartment and that of the drug are pivotal in imparting this selective accumulation. The most important property of the drug is that it contains a weakly basic, ionizable amine functional group that renders the drug relatively membrane-permeable in its unionized form and membrane-impermeable in its ionized form (Figure 1.1A). These properties are described by the acid dissociation constant for the drug (i.e., pKa) and the permeability parameter of the drug known as alpha (α). The important property of the lysosome is its pH, which is maintained at a low level relative to the rest of the cell (Figure 1.1B). Lysosomes are characterized by their acidity which is critical to the endogenous metabolic function of the organelle (Coffey and De Duve 1968). The low luminal pH is maintained through the activity of a membrane-bound protein complex that functions in the ATP-dependent inward pumping of protons (Nelson and Harvey 1999). This protein complex is commonly referred to as the vacuolar-type H⁺-ATPase (V-ATPase).

Upon exposure of cells to the amine-containing drug, the drug is able to diffuse across cellular membranes in its membrane-permeable, unionized form. It is able to enter the various cellular compartments, diffusing down its concentration gradient. Upon entering the highly acidic lysosome the amine-containing drug experiences a dramatic shift in pH and the ionic equilibrium is re-established, highly favoring the membrane-impermeable ionized form of the drug. Because the ionized form of the amine-containing drug is relatively membrane-impermeable and not able to diffuse out of the lysosome, or does so very slowly, it becomes effectively 'trapped' within the compartment (Figure 1.1B). Therefore, this subcellular disposition mechanism would be best described as a passive transport of amine-containing drug into the lysosome that is

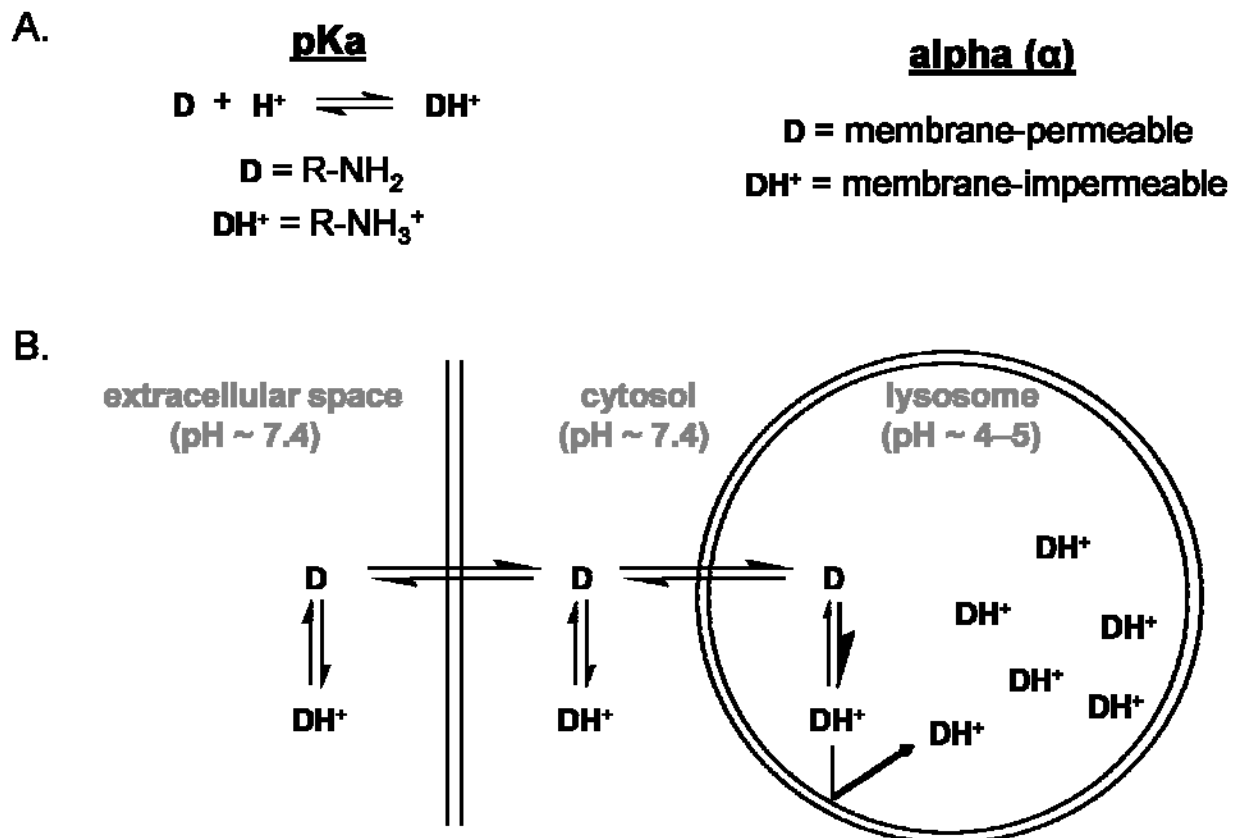


Figure 1.1. Ion trapping mechanism of amine-containing drug accumulation in lysosomes. (A) Physicochemical properties of substrates are described by the presence of a weakly basic ionizable amine ($R-NH_2$), described by its pK_a , and the membrane permeability parameter, α . (B) Diagram of the mechanism of lysosomal ion trapping for weakly basic amine-containing drugs highlighting the pH gradient existing between acidic lysosomes and the extralysosomal compartments. The drug (D) enters the lysosome by passive diffusion and becomes 'trapped' in the lysosome because of the increased formation of membrane-impermeable, ionized drug (DH^+).

facilitated by the activity of the V-ATPase. The importance of the V-ATPase in lysosomal accumulation of these drugs is illustrated by the significant reduction in drug accumulation in lysosomes following treatment with the specific V-ATPase inhibitor, concanamycin A (Figure 1.2).

1.4.2. Physicochemical properties of lysosomotropic amines

Studies into physicochemical predictors of the lysosomotropism of a compound have yielded two key parameters, pKa and alpha (de Duve, de Barsey et al. 1974; Duvvuri, Gong et al. 2004; Duvvuri, Konkar et al. 2005). The alpha value is a measure of the ratio of the octanol:water partition coefficients of the ionized versus unionized form of the drug and is therefore an indicator of the relative membrane permeability of the ionized versus unionized form of the drug. The pKa of the amine-containing drug determines its ionic distribution as a function of pH. This relationship can be best described by the Henderson-Hasselbalch equation where the difference between the pKa of the compound and the pH of the solution is directly related to the logarithm of the fractional ionic distribution. Using an equation derived to approximate the steady state accumulation of amine-containing drugs in lysosomes by ion trapping (Figure 1.3A) an estimate of the lysosome:extracellular concentration ratio of the drug can be predicted based on lysosomal pH ($[H^+]_L$), extracellular pH ($[H^+]_E$), pKa (Ka) and alpha (α) for the amine-containing drug (de Duve, de Barsey et al. 1974).

To first assess the impact of pKa on lysosome:extracellular concentration ratios alpha is set at zero (i.e., the drug is membrane-permeable when unionized and completely membrane-impermeable when ionized), a plot of predicted

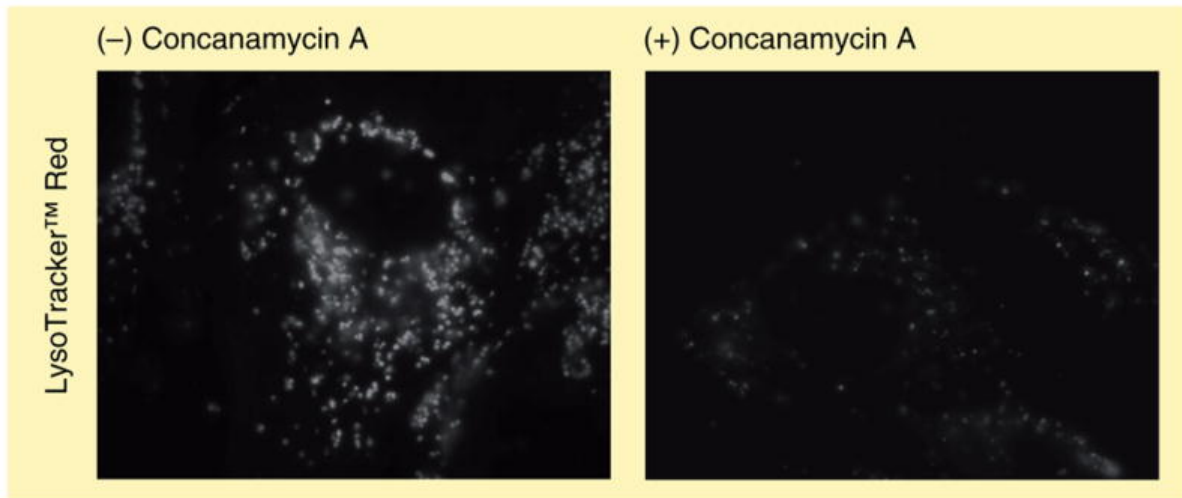


Figure 1.2. Lysosomal drug accumulation is dependent on V-ATPase mediated acidification of lysosomes. The specific inhibitor of the lysosomal V-ATPase, concanamycin A, reduces the lysosomal uptake of LysoTracker Red. LysoTracker Red is a fluorescent amine-containing compound that accumulates by ion trapping in lysosomes. Figure reproduced with permission Future Sciences Ltd (Goldman, Funk et al. 2009).

A.
$$\frac{[lysosome]}{[extracellular]} = \frac{(\alpha[H+]_E + K_a)([H+]_L + K_a)}{([H+]_E + K_a)(\alpha[H+]_L + K_a)}$$

B.

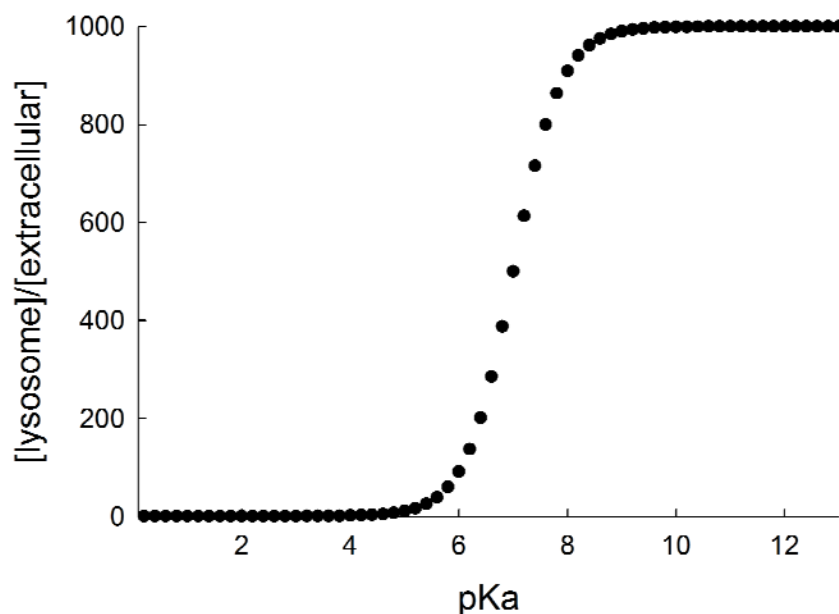


Figure 1.3. Theoretical predictions of the lysosomal accumulation of weakly basic amine-containing compounds by ion trapping. (A) Equation predicting the lysosomal accumulation of weakly basic amine-containing drugs allows predictions of lysosome:extracellular drug concentration ratios ($[lysosome]/[extracellular]$) based on extracellular pH ($[H+]_E$), lysosome pH ($[H+]_L$), pKa of the drug (K_a) and alpha of the drug (α). (B) A graphical representation of the effect of pKa on predicted lysosomal drug accumulation ($[lysosome]/[extracellular]$) using the equation in (A) and assuming: a lysosome pH of 4, extracellular pH of 7 and an alpha of 0.

lysosome:extracellular concentrations is shown with extracellular pH set at 7.0 and lysosomal pH set at 4.0 (Figure 1.3B). As illustrated the ratio of drug concentrations in the lysosome as compared to the extracellular space is highly dependent on pKa with a maximum ratio of 1000 in this plot. The maximum concentration ratio is directly proportional to the ratio of the hydrogen ion concentration in the lysosome relative to the extracellular space. Therefore, a three pH-unit difference results in a predicted concentration ratio of 1000, revealing the potential extent of this trapping phenomenon and the possibility of extremely high local concentrations of drug within the lysosome. Although this hypothetical plot suggests that amines with pKa values greater than 8 will show significant lysosomotropism, it has been argued that as the pKa becomes significantly greater than 8, such a small portion of the drug will exist in the membrane-permeable unionized form that attainment of steady state levels becomes kinetically infeasible with years required to reach steady state (de Duve, de Barsey et al. 1974). Experimental data measuring the effect of pKa on lysosome to cytosol concentration ratios (Duvvuri, Konkar et al. 2005) has confirmed the importance of pKa on lysosomal drug accumulation and shown that compounds up to pKa 9 are still significant substrates for accumulation in lysosomes (Figure 1.4). Although no work has confirmed the hypothetical limited pKa range for optimal lysosomal ion trapping, a targeted pKa to impart lysosomotropic properties in the region of 6 to 10 has since been proposed (Trapp, Rosania et al. 2008).

To assess the impact of the alpha value on predicted lysosome:extracellular concentration ratios for an amine-containing drug a range of alpha values from zero to one were plugged into the equation shown in Figure 1.3A. The resulting plots reveal the

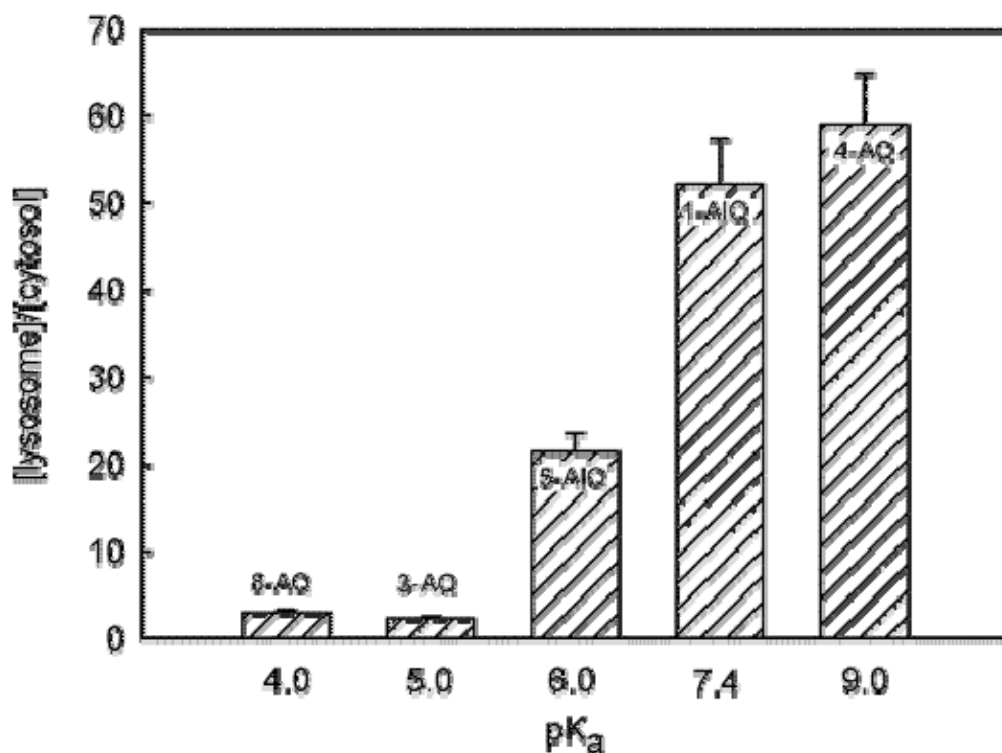


Figure 1.4. Experimental data supporting the relationship between pK_a and lysosomal accumulation of amine-containing compounds. Amine-containing compounds of similar structure but varying pK_a values were assessed for their ability to accumulate in lysosomes ($[lysosome]/[cytosol]$) by measuring lysosome and cytosol drug concentrations from drug treated cells. Lysosomal accumulation increases with pK_a . Reproduced with permission from the American Chemical Society copyright 2005 (Duvvuri, Konkar et al. 2005).

importance of the α value in the predicted lysosome:extracellular concentration ratios (Figure 1.5). As seen, compared to the ideal scenario where α is zero the relationship between pK_a and lysosome:extracellular concentration ratios significantly deviates. This large deviation reflects the increased fraction of the drug in its ionized form with increasing pK_a and its relative ability to permeate cellular membranes as the α approaches one. An α value of one indicates no difference in membrane permeability between the ionized and unionized form of the drug and therefore should completely eliminate the lysosomal ion trapping mechanism.

Experimental measurements of amine-containing drug accumulation in the lysosome relative to the cytosol for compounds of varying α (Duvvuri, Gong et al. 2004) have illustrated that as α approaches one these drugs no longer accumulate in lysosomes (Figure 1.6). In fact, as the α of the amine-containing compounds approached one, they began to accumulate in the mitochondria rather than lysosomes (Duvvuri, Gong et al. 2004). Therefore, these results illustrate the importance of the α value with maximal lysosomotropism, in the form of lysosome:extracellular concentration ratios, predicted to occur as α approaches zero.

1.5. Lysosomal ion trapping in drug development

Although a variety of therapeutic drugs have been either directly (Ishizaki, Yokogawa et al. 2000) or indirectly (Lemieux, Percival et al. 2004; Nadanaciva, Lu et al. 2011) identified as substrates for ion trapping-based accumulation in lysosomes, the subcellular disposition of drugs into lysosomes isn't something routinely considered.

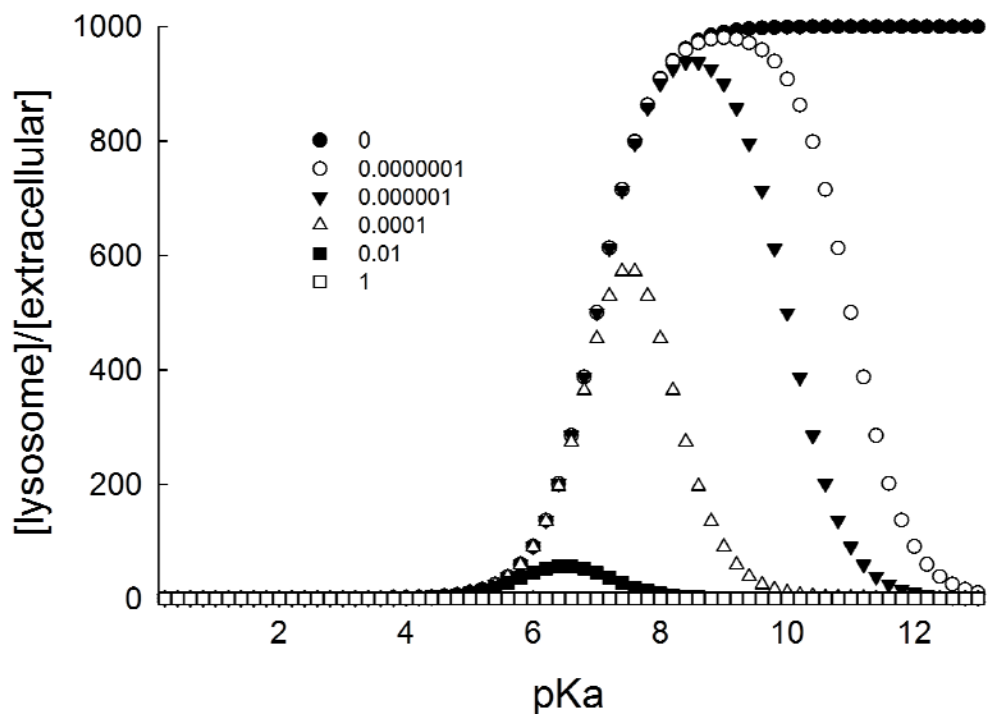


Figure 1.5. The importance of alpha on predicted lysosomotropism of amine-containing drugs. The predicted membrane-permeability of ionized drug relative to unionized drug, represented by alpha, strongly influences the theoretical predictions of drug disposition by lysosomal ion trapping. Predicted lysosomal drug accumulation ($[\text{lysosome}]/[\text{extracellular}]$) as a function of pKa was plotted with alpha values between 0 and 1 (lysosome pH of 4, extracellular pH of 7). Increased alpha results in a predicted decrease in lysosomal ion trapping with no 'trapping' effect observed when alpha is 1.

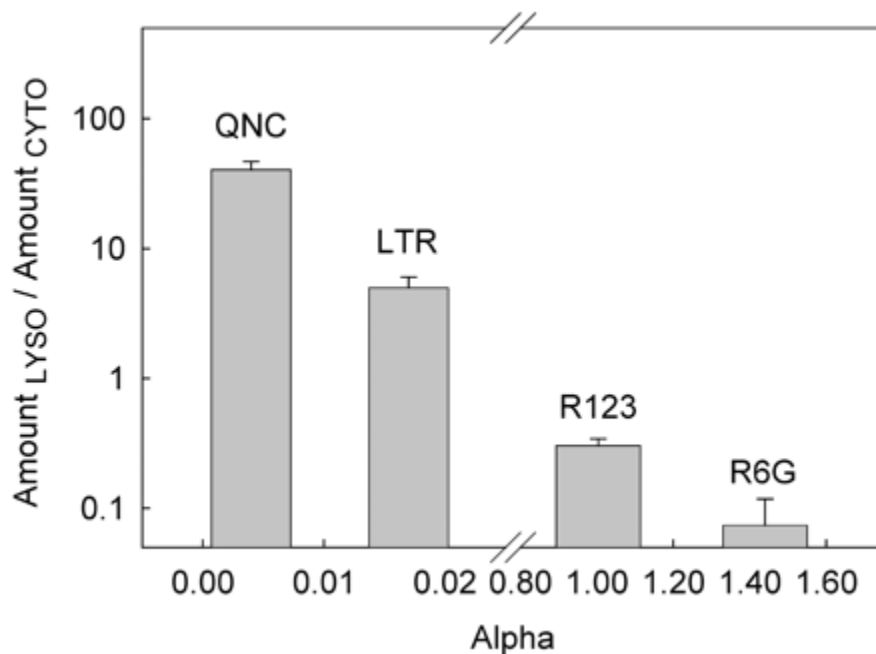


Figure 1.6. Experimental data illustrating the importance of alpha in the lysosomal accumulation of weakly basic amine-containing drugs. Four compounds with pKa values near neutrality (pKa range 7.2 to 8.0), but differing alpha values ranging from 0.004 to 1.440, were assessed for lysosomal accumulation ($\text{Amount}_{\text{LYSO}}/\text{Amount}_{\text{CYTO}}$). Lower alpha values correlated with increased drug accumulation in the lysosome relative to the cytosol with lysosome specific accumulation lost when $\alpha \geq 1$. Reproduced with permission from the American Society for Biochemistry and Molecular Biology (Duvvuri, Gong et al. 2004).

Therefore, the actual prevalence of this drug trapping phenomenon is largely unknown. The speculation for the pervasiveness of this phenomenon is strongly rooted in the understanding of the physicochemical parameters that impart lysosomotropism and the realization that a large portion of drugs fit these parameters. These observations reveal the need for a more thorough understanding of the prevalence of this subcellular disposition phenomenon and its role in the observed pharmacology and toxicology of amine-containing drugs.

Perhaps one of the most obvious implications for drug accumulation in the lysosomes is a high degree of uptake into tissues such as the liver, lungs and kidneys, that are enriched with lysosomes, with lower concentrations in tissues with reduced lysosomal content, such as the heart, skeletal muscle and adipose tissue (Bickel, Graber et al. 1983). The high volume of distribution seen with these drugs is often attributed to lysosomal ion trapping in tissues and is also typically associated with a prolonged half-life which may, or may not, be a desirable pharmacokinetic property depending on the drug and disease being treated. Along with this extensive accumulation in lysosomes many of these drugs have been associated with a toxicological phenomenon known as phospholipidosis (Lullmann-Rauch 1979) which is described by the accumulation of a variety of lipid species, most notably phospholipids, in perinuclear bodies of lysosomal origin (Lullmann, Lullmann-Rauch et al. 1973). Drug-induced phospholipidosis has been attributed to a variety of potential pathologies of the eye (Lullmann-Rauch 1981), kidneys (Lullmann, Lullmann-Rauch et al. 1981), lungs (Cantor, Osman et al. 1984), liver (Shikata, Oda et al. 1970) and immune system (Sauers, Wierda et al. 1988). Despite these finding, phospholipidosis is typically

thought of as a compensatory cellular response to the cellular accumulation of drugs that fails to result in overt organ dysfunction or toxicity (Reasor and Kacew 2001).

Another interesting finding is that the accumulation of therapeutic agents in lysosomes results in a reduction in the pharmacological activity of drugs with extralysosomal targets (Duvvuri, Konkar et al. 2006). Such an observation suggests that the subcellular distribution of drugs can impact their ability to interact with their pharmacological targets and therefore modulates their therapeutic activity. The work in this area has been primarily conducted in anticancer therapeutics and refers to the accumulation of these drugs in lysosomes as an intracellular sequestration phenomenon. It has been found that cancer cells that are able to sequester the weakly basic anticancer agent, daunorubicin, in lysosomes are far less sensitive to the drug than cancer cells that fail to significantly sequester the drug in lysosomes (Duvvuri, Konkar et al. 2005). In fact, one of the mechanisms of drug resistance by cancer cells is thought to be the acquisition of the ability to sequester daunorubicin in lysosomes through a re-acidification of their lysosomes (Gong, Duvvuri et al. 2003; Duvvuri and Krise 2005). In addition, the fact that many cancer cells have an elevated lysosome pH and non-cancerous, normal cells have acidified lysosomes has resulted in a novel drug design strategy whereby anticancer agents are designed to be substrates for lysosomal sequestration (Ndolo, Jacobs et al. 2010). The hypothesis is that non-cancerous cells will be capable of sequestering the drug in non-target containing lysosomes, whereas, cancer cells without acidified lysosomes, will not. This intracellular distribution-based selectivity platform is currently under investigation as a strategy to reduce the activity of

the drug in normal, non-cancerous cells, while maintaining its activity in cancer cells, thus improving the selectivity or therapeutic index of the drug.

1.6. Effect of amine-containing drug accumulation on lysosomes

1.6.1. Amine-containing drugs affect lysosomal structure and function

In considering the importance of lysosomal ion trapping in drug development many have focused on how this subcellular disposition mechanism is important to drug distribution and drug activity. Of great importance is the potential affect of these drugs on the normal physiology of the lysosome, thus playing a potential role in the therapeutic activity or toxicity of these drugs. Realizing that these drugs are predicted to reach concentrations within the lysosome several hundreds to thousands of times that seen in the extracellular space, it would be expected that these drugs would have local effects within the lysosome. A variety of studies utilizing lysosomotropic compounds have illustrated the ability of these drugs to influence lysosomal structure and function (Matsuzawa and Hostetler 1980; Ohkuma and Poole 1981; Poole and Ohkuma 1981; Ohkuma, Chudzik et al. 1986; Morissette, Lodge et al. 2008). Despite the multitude of observations it appears that these effects are most likely secondary to one of three primary effects of amine-containing drugs on lysosomes, including: lysosomal alkalinization, cytoplasmic vacuolization and lysosomal lipid accumulation. All of these effects have been observed with various lysosomotropic compounds and have been directly attributed to the lysosomal accumulation of these drugs. In addition, these effects are believed to result in changes in normal cellular processes that rely on functioning lysosomes.

The following sections will highlight the three main effects of amine-containing drugs on lysosomes and how they result in additional defects in normal lysosomal and cellular functioning. In addition, the hypothesized mechanisms through which these drugs mediate these effects will be discussed along with the observed importance of drug hydrophobicity in differentiating the ability of these drugs to cause these various alterations in lysosome-related properties.

1.6.2. Lysosomal alkalization

Perhaps the most intuitive as well as most mechanistically understood effect of amine-containing drugs on lysosomes is a significant alkalization of the normally highly acidic lysosome (Poole and Ohkuma 1981). The development of lysosomal probes with pH-sensitive fluorescence properties has made this a rather simple parameter to monitor in cell culture models using either ratiometric imaging (Maxfield 1989) or whole cell spectrofluorometric measurements (Ohkuma and Poole 1978). Because of the simplicity of this measurement, amine-induced lysosomal alkalization has been extensively studied and is a well-accepted phenomenon. Measurements of lysosomal pH in cells following treatment with ammonia shows that this effect occurs rapidly following addition of drug, reverses rapidly upon removal of ammonia and is dependent on the dose (Figure 1.7).

The ability of amine-containing drugs to cause a relative alkalization of the lysosome is thought to be a direct result of lysosomal uptake of the amine-containing drug (Ohkuma and Poole 1978). Once the amine-containing drug enters the lysosome it is hypothesized to cause a pronounced decrease in the free hydrogen ion

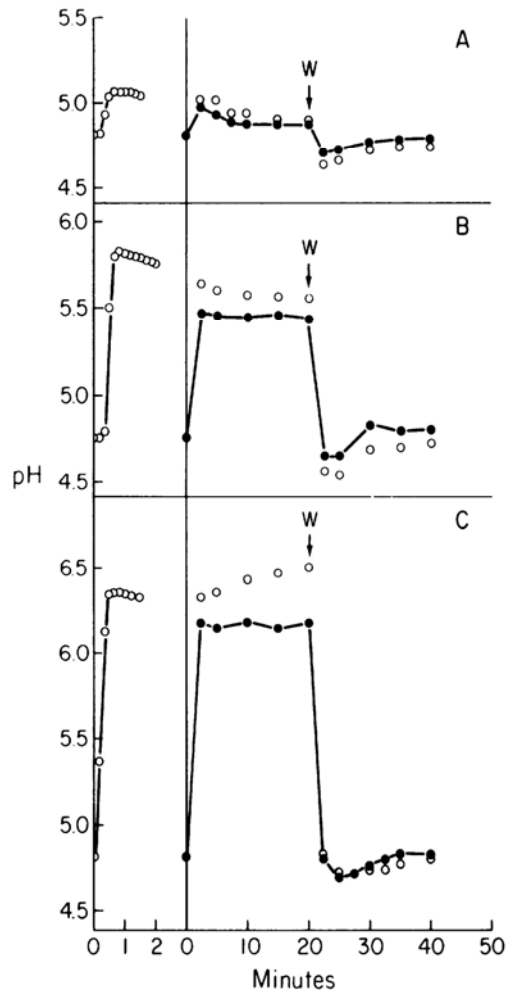


Figure 1.7. Measurements of lysosomal pH using a fluorescence-based assay shows the effect of amine-containing compounds on lysosomal pH in living cells. Lysosome pH at the start of each experiment was below 5.0 and following the addition of (A) 100 μ M (B) 1 mM or (C) 10 mM ammonium chloride the lysosomal pH increased rapidly (< 5 minutes) to a steady state level that significantly depended on dose. At the end of 20 minutes the cells were washed (W) of ammonia and the lysosomal pH rapidly returned to levels at or below its initial value. Open circles represent a non-ratiometric measurement of lysosome pH and closed circles represent a concentration corrected ratiometric measurement of lysosome pH. Reproduced with permission from the National Academy of Sciences (Ohkuma and Poole 1978).

concentration (i.e., increased pH) by either its ability to 1) act as a buffer within the lysosomal lumen or 2) short-circuit the lysosomal pH gradient by transporting hydrogen ions out of the lysosome by diffusing out of the lysosome in its ionized form (Poole and Ohkuma 1981), see illustration in Figure 1.8. In addition, it has also been hypothesized that amine-containing drugs can potentially affect lysosomal pH by inhibiting V-ATPase activity, thereby reducing the transport of protons into the lysosome (Goldman, Funk et al. 2009). Regardless of the mechanism, treatment with weakly basic amine-containing drugs results in a reduced hydrogen ion concentration within the lumen of the lysosome and therefore an increase in pH.

Normally, the acidic lysosomal pH is maintained through the active inward pumping of protons by the V-ATPase, therefore to cause lysosomal alkalinization the rate of proton consumption or transport by the amine drug must exceed the active inward pumping of protons. Intuitively, it might be expected that after prolonged activity of the proton pump, lysosomal pH would rebound resulting in re-acidification, but in vitro time-based studies have not shown that to be the case with no re-acidification of the lysosome occurring over several hours following introduction of drug into the media (Poole and Ohkuma 1981). An ex vivo study assessing the time dependence of chloroquine-induced lysosomal pH perturbations in hepatocytes suggested that treatment with the amine-containing drug results in a transient alkalinization of the lysosome that is reversed within 3 hours of drug administration in rats (Tietz, Yamazaki et al. 1990). The authors speculate that re-acidification of the lysosomes results from the compensatory activity of the V-ATPase, but one must question the role of drug clearance in this apparent reversal of lysosomal alkalinization. Especially since in vitro

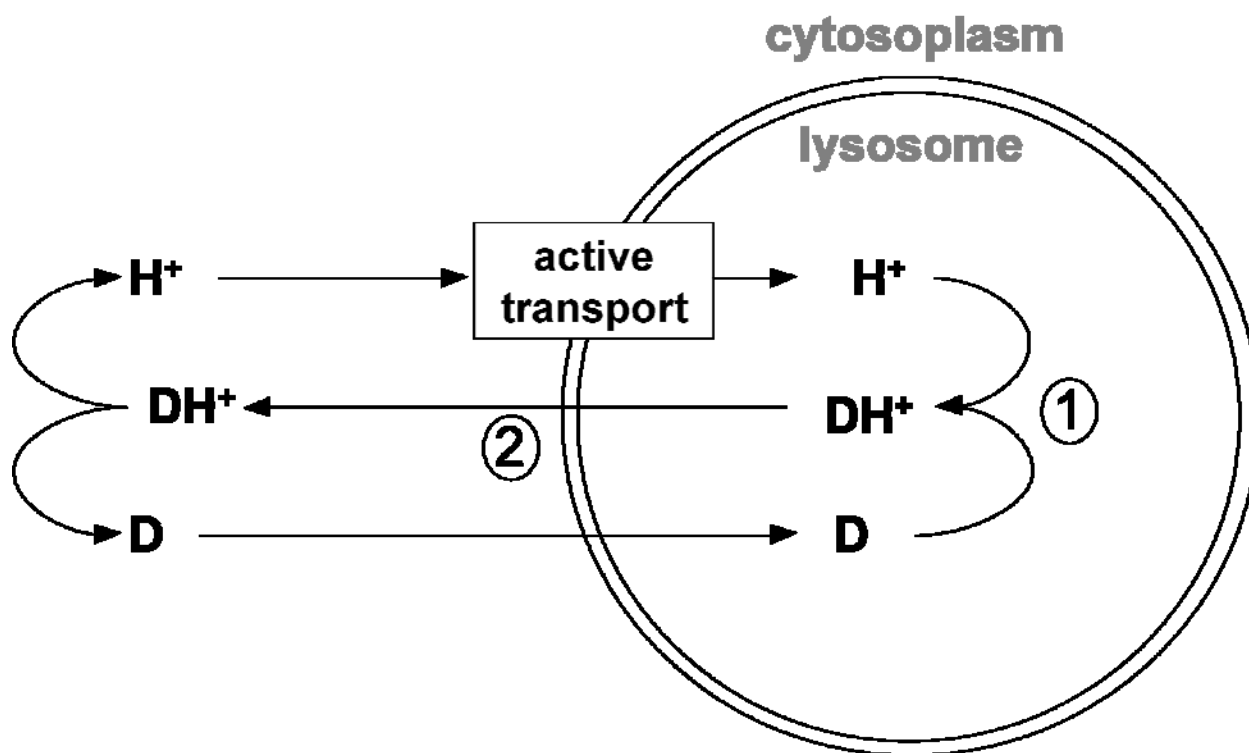


Figure 1.8. Amine-induced lysosomal alkalization. Illustrated are the hypothesized mechanisms by which amine-containing compounds accumulating in the lysosome cause an alkalization of lysosomes. Mechanism 1 is the local buffering effect mediated by the amine-drug entering in its unionized form (B) and consuming free hydrogen ions through its protonation (BH^+). Mechanism 2 is the diffusion of ionized drug (BH^+) out of the lysosome transporting a hydrogen ion with it. Reproduced with permission copyright Poole et al., 1981 (Poole and Ohkuma 1981).

studies have shown that amine-containing drug-induced lysosomal alkalization occurs rapidly following drug exposure and reverses rapidly upon removal of drug (Ohkuma and Poole 1978). Such a rapid effect is expected since the amine-containing drug enters and exits the lysosome through simple passive diffusion, but interestingly it was found that lysosomal pH doesn't directly correlate to cellular uptake of the amine-containing drug (Poole and Ohkuma 1981). This effect was illustrated using the highly lysosomotropic amine, methylamine, where the initial uptake phase for methylamine appears to correspond to a rapid elevation in lysosomal pH but a subsequent secondary uptake phase ensues that doesn't correspond to any further change in lysosomal pH (Figure 1.9). This apparent lack of direct correlation between uptake of lysosomotropic amines and changes in lysosomal pH makes one question the information that can be obtained from recently developed assays that measure the lysosomotropic properties of drugs based on their ability to cause lysosomal alkalization (Lemieux, Percival et al. 2004; Nadanaciva, Lu et al. 2011). These assays may provide a more qualitative than quantitative determinant of a drug's lysosomotropic properties and may make direct comparisons of lysosomotropism between drugs quite difficult.

Interestingly, different amine-containing compounds have a very large difference in their potency in causing lysosomal alkalization (Poole and Ohkuma 1981). Investigations into the structural predictors of what causes one amine-containing drug to be more potent at causing lysosomal alkalization than a second resulted in a relationship between drug hydrophobicity and potency in causing lysosomal alkalization (Ishizaki, Yokogawa et al. 2000). Lysosomal pH was measured in isolated lysosomes treated with several amine-containing drugs (Figure 1.10). The potency of

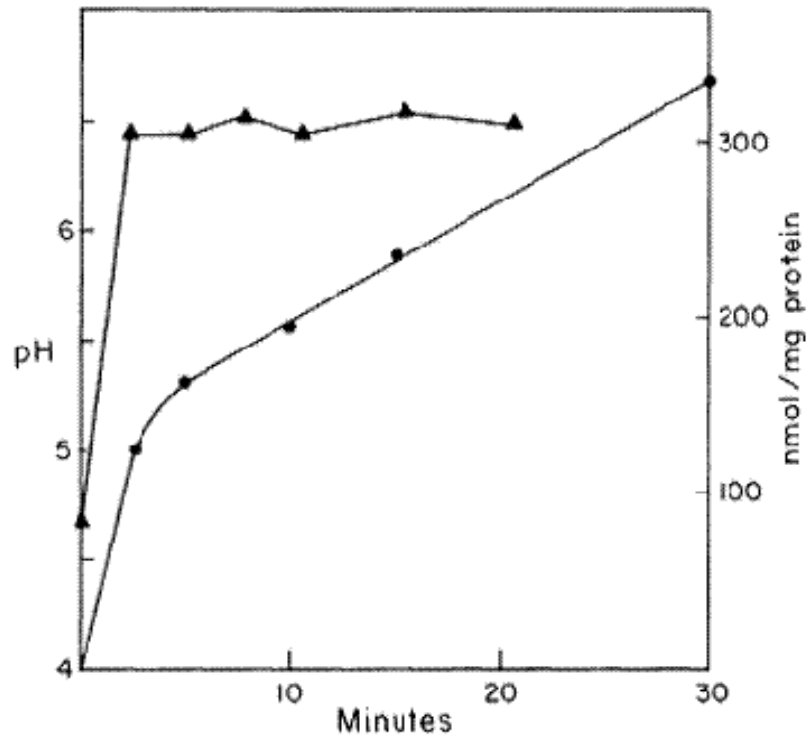


Figure 1.9. Lysosomal alkalization by the amine containing compound, methylamine, doesn't directly correlate to cellular uptake of the drug. Time-based measurements of lysosome pH (triangles) and cellular uptake of methylamine (circles) after the addition of 10 mM methylamine to cells in culture shows a rapid alkalization of lysosomes. Initial pH values are below 5.0 and reach a steady state level (pH 6.5) in less than 5 minutes following addition of drug. Cellular uptake of methylamine appears to be biphasic with an initial uptake phase that correlates to the observed pH change, and a secondary uptake phase that continues through the remainder of the experiment with no further change in lysosome pH. Reproduced with permission copyright Poole et al., 1981 (Poole and Ohkuma 1981).

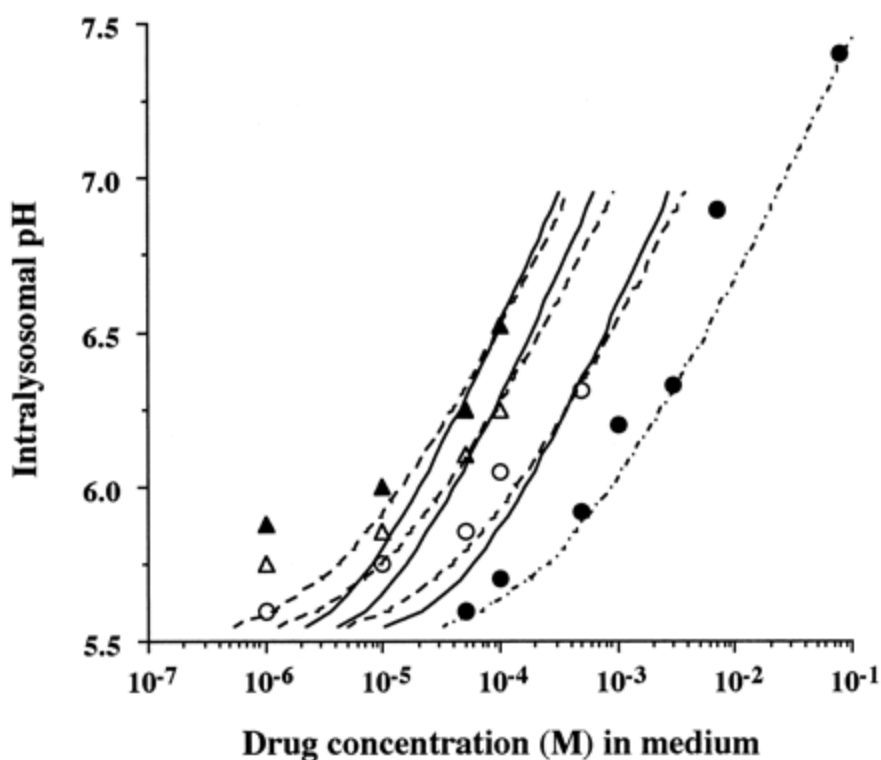


Figure 1.10. Measurement of pH in isolated lysosomes following treatment with weakly basic amine-containing drugs of varying hydrophobicity. Isolated lysosomes were treated with dose titrations of chlorpromazine (closed triangles), imipramine (open triangles), propranolol (open circles) and ammonium chloride (closed circles). Dose-relationships for alkalinization of lysosomes showed that the potency of these compounds (chlorpromazine > imipramine > propranolol > ammonium chloride) correlated to their relative hydrophobicity (chlorpromazine > imipramine > propranolol > ammonium chloride) as determined by logP values which were determined using the logP plug-in from the MARVIN software freely available at www.chemaxon.com. Reproduced with permission from (Ishizaki, Yokogawa et al. 2000).

these compounds in causing lysosomal alkalization (chlorpromazine > imipramine > propranolol > ammonium chloride) appeared to correlate to their relative hydrophobicity (chlorpromazine > imipramine > propranolol > ammonium chloride).

Subsequent studies that indirectly measured lysosomal alkalization by measuring inhibition of imipramine uptake into isolated lysosomes found a similar relationship (Figure 1.11). Concentrations of drug necessary to inhibit lysosomal uptake of imipramine by 50% (i.e., IC_{50}) were plotted as a function of the hydrophobicity of the drug (i.e., logP). Increased hydrophobicity correlated with a decreased IC_{50} , indicating that hydrophobic drugs were more potent lysosomal alkalizers than their relatively hydrophilic counterparts. In addition, it was found that amine-containing drugs with increased hydrophobicity accumulate in lysosomes to a much higher degree as compared to the hydrophilic ones (Ishizaki, Yokogawa et al. 2000). The enhanced capacity of lipophilic amines to accumulate in lysosomes and cause lysosomal alkalization was proposed to result from intralysosomal binding and/or aggregation of the ionized lipophilic amines which facilitates the enhanced lysosomal accumulation of the amine-containing drugs.

The acidic environment of lysosomes is an important physiological characteristic that facilitates many of the organelle's biological functions. Many of the lysosomal enzymes are known to be optimally active under acidic conditions and to require activation within the acidic environment of the lysosome in order to be processed from their inactive pro-forms into their active forms (Kornfeld 1986). Therefore, amine-induced alkalization of lysosomes results in both a decrease in the hydrolytic activity of lysosomal enzymes (Ohkuma, Chudzik et al. 1986) and a defect in the normal

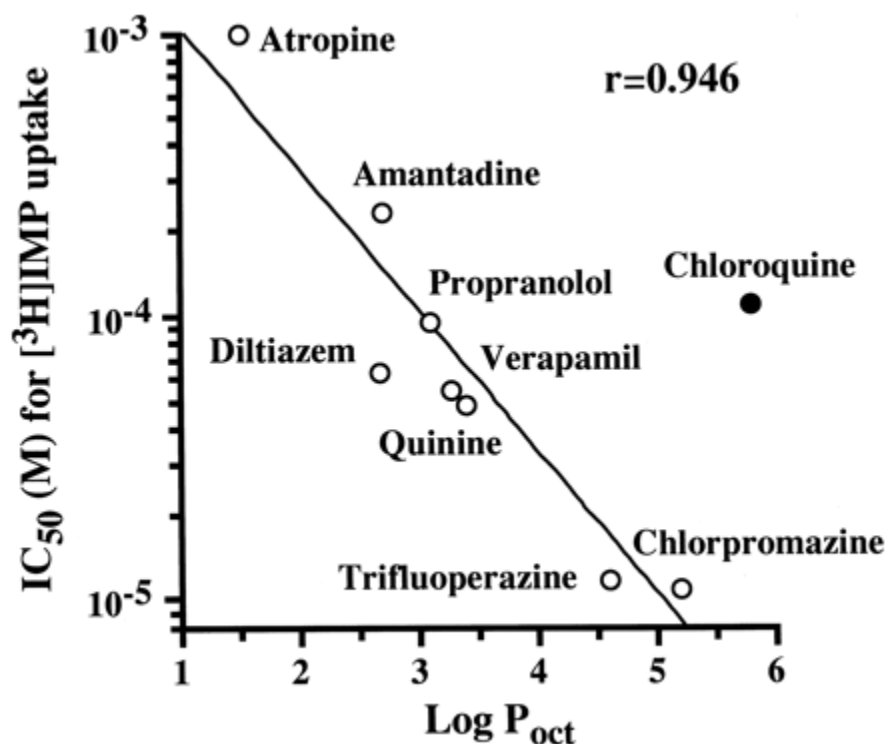


Figure 1.11. Potency in causing alkalinization of lysosomes correlates with drug hydrophobicity. A series of amine-containing drugs were experimentally evaluated for the concentration necessary to inhibit lysosomal uptake of imipramine by 50% (IC_{50}). Inhibition of imipramine uptake is assumed to be directly related to alkalinization of lysosomes. Regression analysis of the plot of IC_{50} versus hydrophobicity ($\text{Log } P_{\text{oct}}$) shows a strong correlation. Drugs of increased hydrophobicity appear to be more potent at causing lysosome alkalinization. Reproduced with permission (Ishizaki, Yokogawa et al. 2000).

processing of lysosomal enzymes (Cardelli, Richardson et al. 1989). Additionally, the pH of the lysosome has been shown to be an important regulator of lysosomal calcium concentrations with amine-induced lysosomal alkalization resulting in decreased lysosomal calcium levels (Christensen, Myers et al. 2002). Under the normal processing of ligands for receptor-mediated endocytosis, the acidic environment of endocytic compartments is instrumental in the dissociation of receptor-ligand complexes; therefore, amine-induced perturbations in endocytic acidification results in the accumulation of un-dissociated complexes that either accumulate in the endocytic compartment (Harford, Wolkoff et al. 1983) or recycle back to the cell surface (Ciechanover, Schwartz et al. 1983).

Although few drugs are known to cause significant lysosomal alkalization at therapeutically relevant concentrations, the toxicological and pharmacological implications of such effects have been hypothesized and tested in various systems. The anti-malarial chloroquine is perhaps the most studied drug that is known to cause a significant lysosomal alkalization at drug concentrations reached in vivo and has had many of its pharmacological properties attributed to this unique effect. Since chloroquine is a dibasic compound with two ionizable amines with near neutral pKa values (Hollemaans, Elferink et al. 1981), its lysosomotropic properties far exceed those of mono-basic amines, and thus, results in an extremely high level of lysosomal accumulation and alkalization that is observed at therapeutically relevant drug concentrations. The anti-malarial activity of chloroquine relies heavily on its ability to accumulate in the acidic food vacuole of the malaria parasite, but is not mediated by its alkalizing effect (Ginsburg, Nissani et al. 1989). However, pharmacological activity of

chloroquine as an anti-viral and anti-inflammatory agent has been more closely linked to its ability to cause lysosomal alkalinization (Savarino, Boelaert et al. 2003). Studies using other amine drugs have further suggested the potential therapeutic utility of lysosomal alkalinization in the treatment of viral infections, through inhibition of viral escape from endocytic compartments and/or the intracellular processing of viral proteins (Baxt 1987; Ashfaq, Javed et al. 2011).

In addition to the pharmacological utility of lysosomal alkalinization, other studies have indicated potential toxicological ramifications. For example, the lysosomal alkalinizing amine, ammonium chloride, was found to cause the intracellular accumulation of inclusion bodies similar to those seen in Parkinson's disease with the selective loss of dopaminergic and noradrenergic neurons resulting in movement disorders in medaka fish (Matsui, Ito et al. 2010). This work suggested that environmental toxins with lysosomotropic properties may be pathogenic mediators of Parkinson's disease, but would also include many therapeutic agents because of their lysosomotropic properties. Another interesting study that was born out of the association of methamphetamine use with advanced progression of AIDS revealed that the lysosomal alkalinizing effect of methamphetamine results in immunosuppressive effects in vitro (Talloczy, Martinez et al. 2008). At pharmacologically relevant concentrations, methamphetamine exposure resulted in defective antigen processing and presentation, as well as, reduced phagocytic activity and increased intracellular proliferation of fungi that are common mediators of secondary disease in HIV+ patients. The hypothesis that methamphetamine mediates its immunosuppressive activity through lysosomal alkalinization was supported by illustrating the ability of chloroquine-

induced lysosomal alkalinization to cause the same immunosuppressive phenotype in vitro.

Because lysosomal pH is a large driving force for the cellular and tissue accumulation of various drugs it plays a significant role in the tissue-distribution and pharmacokinetic properties of these drugs, therefore factors that influence the accumulation of these drugs in lysosomes would be expected to result in altered pharmacokinetic properties. One such example would be the co-administration of these drugs with other compounds that cause alkalinization of lysosomes, resulting in reduced drug uptake into lysosomes. In fact, a series of studies has illustrated that drugs causing lysosomal alkalinization result in decreased lysosomal uptake of secondarily administered lysosomotropic amine drugs (Daniel 2003). Reduced lysosomal uptake results in decreased drug uptake into tissues enriched with lysosomes (i.e., lysosome-rich) and increased drug concentrations in tissues lacking in lysosomal content (i.e., lysosome-poor). The significance of this interaction is seen with neuroleptic agents that accumulate in lysosomes. These agents mediate anticholinergic toxicity through their interaction with the lysosome-poor cardiac tissue. Therefore, co-administration of the neuroleptic agents with drugs known to cause lysosomal alkalinization would result in decreased drug uptake in lysosome-rich tissue and increased drug concentrations in the lysosome-poor cardiac tissue. Increased concentrations in the cardiac tissue would be expected to facilitate an increased risk for anticholinergic cardiotoxicity (Daniel and Wojcikowski 1999) and would represent a significant pharmacokinetic drug-drug interaction.

1.6.3. Cytoplasmic vacuolization

Even before lysosomal ion trapping was recognized as a subcellular disposition phenomenon, it was realized that a variety of drugs can induce an apparent expansion of the vacuolar compartment that was referred to as cytoplasmic vacuolization (Yang, Strasser et al. 1965). Later studies sought to reveal the mechanism of this apparently non-toxic, gross morphological effect on cells. It was shown that the amine-containing compound, methylamine, causes the development of large cytoplasmic vacuoles that decrease in size when methylamine accumulation in lysosomes is reduced by either decreasing the pH of the culture medium or reducing its concentration (Figure 1.12).

Similarly, comparison of the cellular uptake of several amine-containing compounds and the resulting appearance of cytoplasmic vacuoles indicated that vacuolization was directly dependent on cellular uptake of the drug (Figure 1.13). Following exposure to drugs at various concentrations cytoplasmic vacuolization was assessed by microscopy and scored based on intensity, with three being major vacuolization and zero being no vacuolization. These results suggested that cytoplasmic vacuolization was secondary to the intense lysosomal accumulation of weakly basic amine-containing drugs by an ion trapping-based mechanism (Ohkuma and Poole 1981).

Further studies using the weakly basic amine-containing drug, procainamide (Morissette, Moreau et al. 2004; Morissette, Lodge et al. 2008), have found that amine-drug accumulation in lysosomes results in a time-dependent osmotic swelling of the lysosome that is reversible upon removal of drug and can be inhibited by either treatment with the V-ATPase inhibitor bafilomycin A1 or co-treatment with the

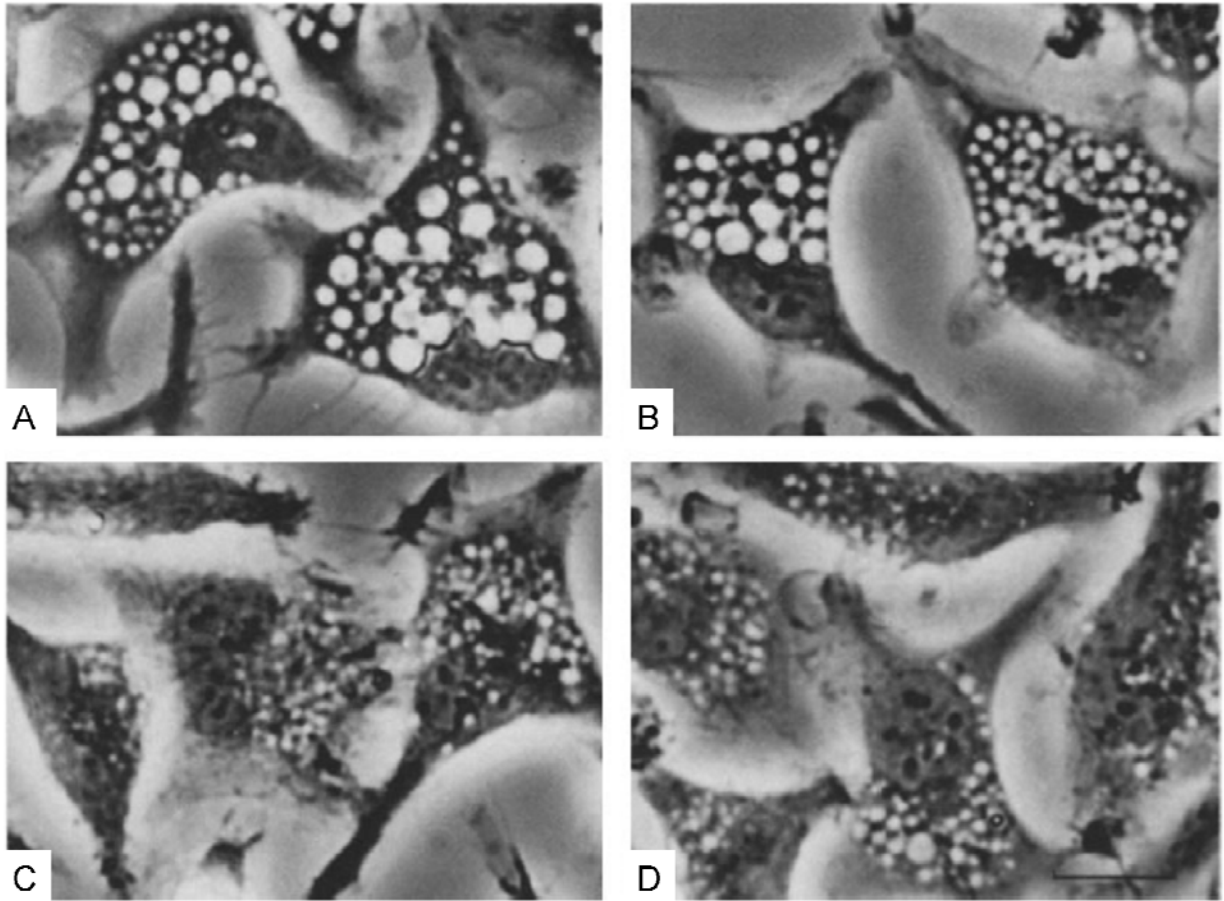


Figure 1.12. Cytoplasmic vacuolization following methylamine treatment depends on pH of the media and methylamine concentration. Addition of 10 mM methylamine to cells at (A) pH 7.6, (B) pH 7.0 or (C) pH 6.6 observed by phase microscopy shows an intense vacuolization that decreases with decreases in media pH. (D) 1 mM methylamine at pH 7.6 shows a reduced level of vacuolization as compared to (A). These results support a lysosomal ion trapping dependent induction of cytoplasmic vacuolization by methylamine. Reproduced with permission copyright Poole et al., 1981 (Ohkuma and Poole 1981).

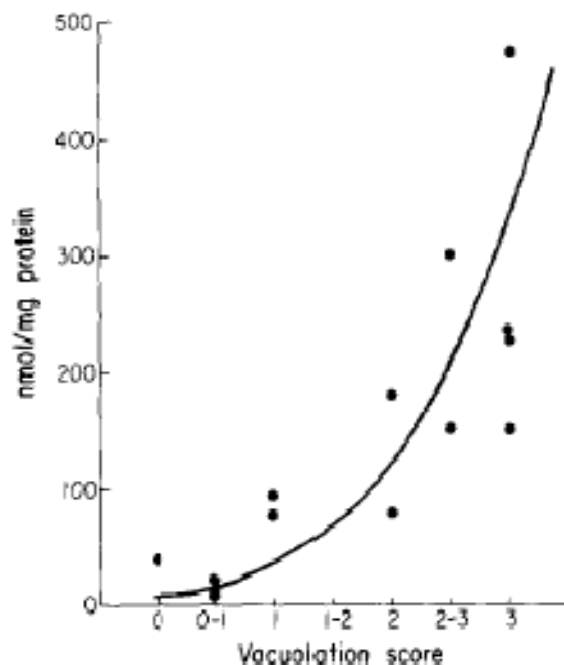


Figure 1.13. Cellular uptake of weakly basic amine-containing compounds is associated with cytoplasmic vacuolization. Measurement of cellular uptake of a variety amine-containing compounds in nmol/mg of protein is plotted as a function of the vacuolization score determined following drug treatment by phase microscopy. A score of 0 indicates no vacuolization and 3 is the most intense vacuolization. A high level of cellular uptake of the compound appears to be necessary to cause vacuolization. Reproduced with permission copyright Poole et al., 1981 (Ohkuma and Poole 1981).

membrane-impermeable osmotically active sugar, mannitol (Figure 1.14). The procainamide-induced cytoplasmic vacuoles were characterized by the presence of a variety of cellular organelle markers, including: lysosomes, late endosomes and the Golgi apparatus. The presence of these various organelle markers led to the hypothesis that cytoplasmic vacuolization results from the fusion of various cellular compartments resulting in enlarged hybrid organelles. Similarly, studies of amine-induced vacuolization have shown that the lysosomal membrane protein, NPC1, is important in the regulation of the fusion of late endosomes and lysosomes in the formation of the amine-induced vacuoles (Kaufmann and Krise 2008), therefore despite the role of osmotic pressure in the formation of vacuoles the fusion of intracellular compartments is critical in the formation of the vacuoles. In addition, it was found that the cytoplasmic vacuoles labeled with autophagic markers, indicating a role for the autophagic stress response in drug-induced vacuole formation (Morissette, Lodge et al. 2008). The proposed mechanism of drug-induced cytoplasmic vacuolization is illustrated in Figure 1.15. Lysosomal accumulation of the amine-drug increases the tonicity of the lysosomal lumen resulting in the osmotic influx of water. Through the fusion of lysosomes with other cellular organelles, grossly enlarged hybrid organelles are formed (i.e., cytoplasmic vacuoles) containing the drug and water.

Studies investigating drug-induced vacuolization with amine-containing compounds have documented the ability of a variety of relatively hydrophilic amine-containing compounds ($\log P < 2$) to cause a pronounced cytoplasmic vacuolization marked by the accumulation of large translucent vacuoles, whereas, the ability of amine-containing drugs of relative hydrophobicity ($\log P > 2$) rarely cause this effect

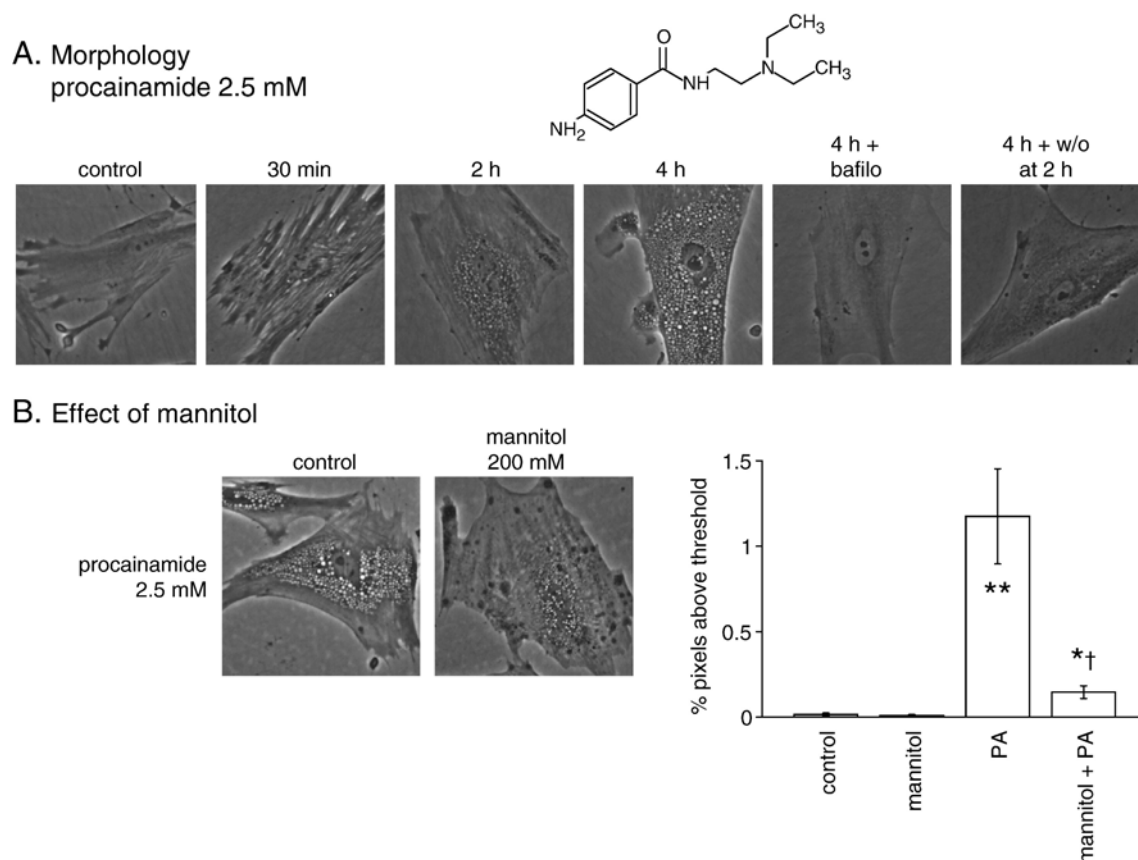


Figure 1.14. Cytoplasmic vacuolization induced by the weakly basic amine, procainamide (PA), is a reversible process that is time dependent, osmotically driven and dependent on lysosomal uptake of drug. (A) Cells treated with procainamide (structure shown) at a concentration of 2.5 mM for up to 4 hours show a time dependent development of cytoplasmic vacuoles as shown by phase microscopy. Addition of the V-ATPase inhibitor, bafilomycin A1 (4h+bafilo), prevents the formation of vacuoles. Removal of procainamide with a 2-hour washout (4h+w/o at 2h) reversed the vacuolization. (B) Addition of the membrane-impermeable osmotically active sugar, mannitol, prevented the procainamide-induced vacuolization as seen by phase microscopy with quantitative analysis of the images measuring vacuolization as pixels above threshold. Reproduced with permission from Elsevier (Morissette, Lodge et al. 2008).

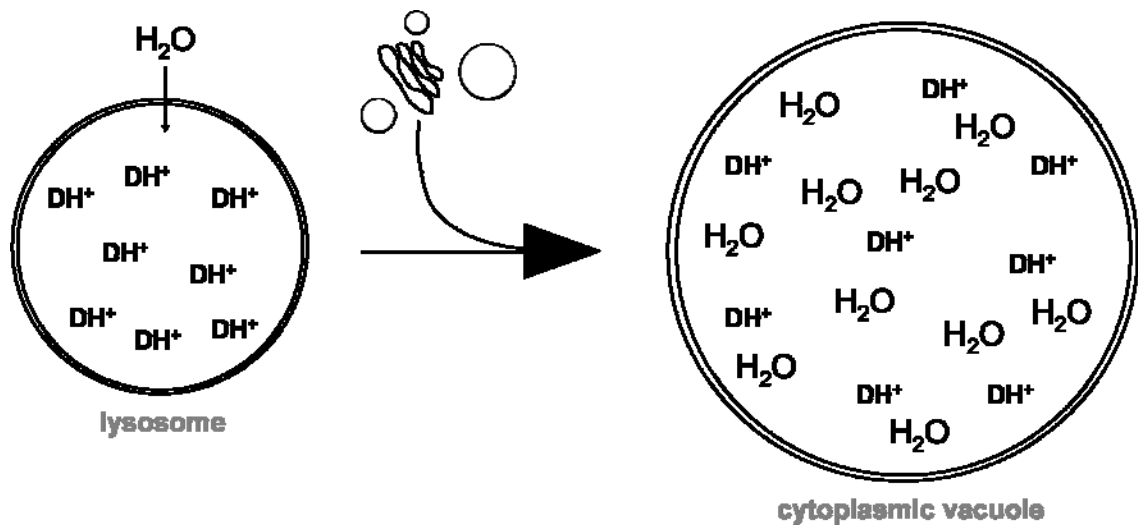


Figure 1.15. Amine-induced cytoplasmic vacuolization. Illustrated is the hypothesized mechanism by which amine-drugs (DH^+) accumulating in the lysosome cause the development of cytoplasmic vacuoles. Amine-drugs accumulating in lysosomes facilitate the osmotic influx of water resulting in grossly enlarged cytoplasmic vacuoles. Vacuoles form through the recruitment of organelle membrane components from a variety of organelles including lysosomes, endosomes and the Golgi apparatus.

(Ohkuma and Poole 1981). Although both hydrophilic and hydrophobic amine-containing compounds accumulate in lysosomes, the reason why one group appears to cause vacuolization while the other does not remains unclear. These observations led early investigators to speculate that amine-containing drugs that do not cause cytoplasmic vacuolization fall into one of four categories that may help explain this observation (Figure 1.16). Group 2 and 3 compounds are proposed to be poor substrates for lysosomal ion trapping because of their low pKa or poor membrane permeability, respectively. Without significant accumulation in lysosomes, cytoplasmic vacuolization can not occur. Group 1 and 4 compounds on the other hand are proposed to be significantly lysosomotropic but because of their hydrophobicity or toxicity are not capable of causing vacuolization. Group 1 compounds are thought to be hydrophobic, even in their ionized state and therefore able to readily diffuse out of lysosomes in their ionized form resulting in dissipation of lysosome pH but insufficient lysosomal concentrations of drug necessary to cause vacuolization. Group 4 compounds, including the well-known hydrophobic amine-drug chlorpromazine, are thought to be toxic to cells at concentrations below those necessary to result in cytoplasmic vacuolization. The reason hydrophobic amine-drugs are not typically associated with cytoplasmic vacuolization requires further elucidation. Since cytoplasmic vacuolization is proposed to be directly related to the lysosomal uptake of the drug, perhaps measurements of lysosomal drug levels would help answer this question.

Although lysosomal volume expansion through the induction of a cytoplasmic vacuolization is an interesting in vitro observation, it often requires exposure to

TABLE II
Basic Substances That Do Not Cause Vacuolation at
Concentrations Up to 10 mM

Group	Compound	pK _a (s)	Refer- ence	mol wt
1	S-Collidine	7.4	(16)	121
	Benzylamine	9.3	(16)	107
	Tributylamine	9.9	(16)	185
2	β-naphthylamine	4.2	(16)	143
	Aniline	4.6	(16)	93
	Pyridine	5.2	(16)	79
	N,N-Diethylamine	6.6	(16)	149
3	Triethanolamine	7.8	(16)	149
	Tris (hydroxymethyl)- aminomethane	8.1	(16)	121
		6.1, 9.9	(16)	111
	Histamine	8.3, 9.8,	(15)	145
	Spermidine	10.9	(14)	202
	Spermine	8.0, 8.9,	(16)	59
	Guanidine	10.0, 10.9 13.6		
4	Chlorpromazine	9.3	(16)	319
	9-Aminoacridine	10.0	(16)	194
	Quinacrine	7.7, 10.2	(16)	400

Figure 1.16. Amine-containing compounds that fail to induce a significant cytoplasmic vacuolization: importance of hydrophobicity. Compounds that fail to cause vacuolization are divided into 4 groups based on the proposed reasoning. Group 2 and 3 are proposed not to be ideal substrates for lysosomal ion trapping because of their pK_a or poor membrane permeability, respectively. Group 1 and 4 compounds include a number of relatively hydrophobic amines and are hypothesized to be too hydrophobic or too cytotoxic to cause vacuolization, respectively. These results suggest hydrophobicity of the amine-containing compound may be an important predictor of the ability of a drug to cause vacuolization with drugs of increased hydrophobicity unable to cause vacuolization. Reproduced with permission copyright Poole et al., 1981 (Ohkuma and Poole 1981).

extremely high (i.e., millimolar) concentrations of amine-containing drug and is therefore of questionable therapeutic significance. In addition, amine-induced vacuolization has not been observed in vivo and may be strictly an in vitro phenomenon. Studies into the role of cytoplasmic vacuolization in the pharmacological activity of various drugs have been hypothesized for local anesthetics (Bawolak, Morissette et al. 2010) and topical anti-wrinkle cosmeceuticals (Morissette, Germain et al. 2007). One issue is the ability to differentiate the effect of vacuolization versus lysosomal alkalization since both are known to occur at these extreme drug concentrations. Some evidence has suggested that cytoplasmic vacuolization inhibits the cellular secretory pathway and mediates cytotoxicity independent from the drug-induced effects on lysosomal pH (Morissette, Moreau et al. 2005). This lack of supportive evidence necessitates further studies to illustrate the potential therapeutic relevance of amine-induced vacuolization before this off-target effect is seriously considered as a pharmacological mediator of drug activity or toxicity.

1.6.4. Lysosomal lipid accumulation

Arguably the most therapeutically relevant of the drug-induced alterations in normal lysosomal physiology is the inhibition of normal lysosomal lipid metabolism. In fact, this phenomenon is routinely screened for in pre-clinical drug development. The discovery of this drug-induced syndrome came after a number of clinical cases of an idiopathic syndrome that was identified simply as a “foam cell syndrome” marked by the cellular accumulation of lipids. These cases were found to coincide with the chronic administration of the drug, 4-4'-diethylaminoethoxyhexesterol (Yamamoto, Adachi et al.

1971). The syndrome was described by a variety of findings, including hepatosplenomegaly, hyperlipidemia as well as the observation of intracellular inclusion bodies by electron microscopy. The drug was found to cause a systemic drug-induced lipidosis that resulted in the tissue accumulation of cholesterol, cholesterol esters, triglycerides and phospholipids (Yamamoto, Adachi et al. 1971). Further studies found this effect to be associated with a number of drugs from differing pharmacological classes (Lullmann, Lullmann-Rauch et al. 1973); since phospholipids appeared to be the primary lipid species involved, this effect became commonly referred to as a drug-induced phospholipidosis. Electron microscopic studies with the lipidosis-inducing drug, AY-9944, revealed the presence of discrete intracellular inclusion bodies that have a distinct multilamellar structure (Figure 1.17) similar to those seen in cells from patients suffering from genetic errors in lipid metabolism (Sakuragawa 1976). These inclusion bodies are often referred to as multilamellar bodies (MLBs) and are thought to be of lysosomal origin as evidenced by positive staining for the lysosomal enzyme, acid phosphatase. Observations of this drug-induced phenotype with significant similarity to the enigmatic hereditary lysosomal storage disease, Niemann-Pick disease, led to the suggestion that an understanding of the pharmacology of the drug may allow for an understanding of the pathogenesis of the disease (Sakuragawa, Sakuragaw et al. 1977). More recently these drugs have been used as pharmacological inducers of the Niemann-Pick disease phenotype in a variety of in vitro models (Roff, Goldin et al. 1991).

Cellular fractionation studies following treatment with the lipidosis-inducing drugs, chloroquine or 4-4'-diethylaminoethoxyhexesterol, allowed for the further confirmation of

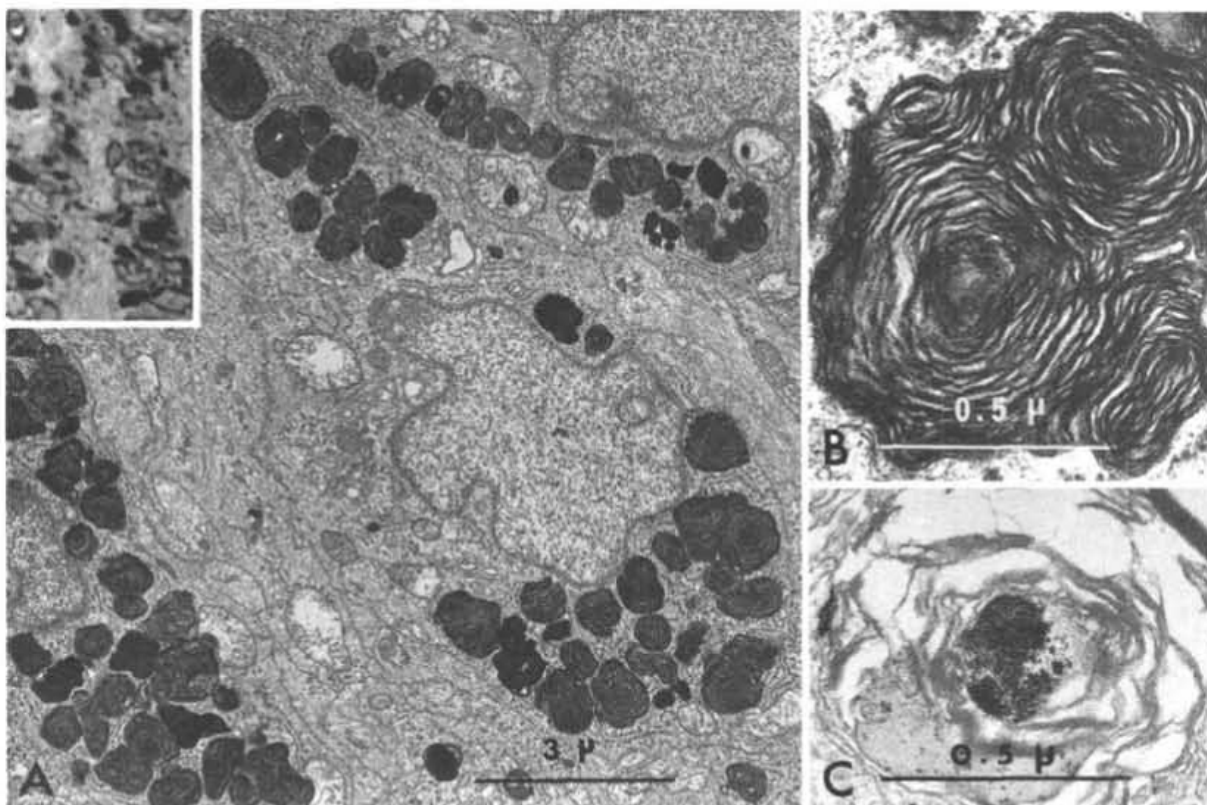


Figure 1.17. The lipodosis-inducing drug AY-9944 causes the accumulation of intracellular inclusion bodies of lysosomal origin. Inset is a light microscopic image of a tissue section from the optic nerve of a rat following repeated injections of AY-9944. Electron microscopic images showing (A) cytoplasmic accumulation of inclusion bodies in glial cells. Increased magnification images of intracellular inclusion bodies are described as having (B) a lamellar structure, often referred to as a multilamellar body (MLB), and (C) positive staining for the lysosomal marker enzyme, acid phosphatase. Reproduced with permission from the Association for Research in Vision and Ophthalmology (Sakuragawa 1976).

MLBs as organelles of lysosomal origin that are enriched in phospholipids, cholesterol and, importantly, the inducing drug (Matsuzawa and Hostetler 1980). Such observations of drug and lipid accumulation in the same organelle have supported the hypothesis that these drugs are active within the lysosome and inhibit lysosomal lipid metabolism by either interacting with the lipids or the enzymes responsible for their metabolism. NMR studies on the interaction of a variety of these drugs with phosphatidylcholine suggested that phospholipids, with charged groups, interact with these drugs in a manner that depends on the relative hydrophobicity of the drug and the presence of a cationic side group (Seydel and Wassermann 1976). The complex has been hypothesized to include hydrophobic interactions between the drug and lipid, as well as, charge-charge interactions between the cationic drug and the anionic portion of the lipid. These findings support the hypothesis that formation of drug-lipid complexes inhibits the enzymatic degradation of these lipids (Lullmann, Lullmann-Rauch et al. 1975).

Others have hypothesized that lipidosis-inducing drugs bind the lysosomal hydrolases and directly inhibit their ability to hydrolyze the various lipids (Martin, Kachel et al. 1989). Of interest though is the apparent lack of ability of these drugs to inhibit lipid metabolism in reconstituted assays using cell homogenates. In studies that measured the inhibition of the lysosomal hydrolase, acid sphingomyelinase (ASMase) (Yoshida, Arimoto et al. 1985), it was found that the lipidosis-inducing drug, AY-9944, specifically inhibited the cellular activity of ASMase (Figure 1.18). AY-9944 inhibited the enzymatic activity of ASMase as indicated by metabolism of sphingomyelin and the two marker substrates: HNP and Bis(4-MU)phosphate. Activity measurements of a

Enzyme Substrates	Enzyme Activities	
	Control (n=3)	AY-9944 (n=6)
Phosphodiesterase		
[¹⁴ C]Sphingomyelin	180±4.6	96.2±13.3
HNP	297±34.7	181±18.6
Bis(4-MU)phosphate	156±11.7	102±6.12
Acid phosphatase	1260±43.6	1350±113
β-Glucosaminidase	10510±874	12190±990
α-Galactosidase	54.3±4.55	58.8±5.09
β-Galactosidase	697±15.3	768±64.7
α-Mannosidase	41.2±9.78	38.9±3.48

Figure 1.18. Activity of various lysosomal hydrolase enzymes in the postnuclear supernatant following the treatment of cells with the lipidosis-inducing drug, AY-9944. Compared to control, untreated cells, the cells treated with AY-9944 showed a significant reduction in acid sphingomyelinase activity (phosphodiesterase) as monitored by the hydrolysis of several substrates including carbon-14 labeled sphingomyelin. Of the various hydrolase activities measured only acid sphingomyelinase activity was significantly reduced. The lipidosis-inducing drug did not appear to non-specifically inhibit lysosomal hydrolase activity. Reproduced with permission Oxford University Press (Yoshida, Arimoto et al. 1985).

variety of other lysosomal hydrolases however revealed no change following AY-9944 treatment. These results suggested that the reduced activity was enzyme specific and did not result from a generalized reduction in lysosomal hydrolase activity.

Interestingly, these authors also found that addition of AY-9944 to cellular homogenate resulted in no direct inhibition of ASMase activity. Inhibition of ASMase appeared to require exposure of living cells to the AY-9944 prior to the homogenization process in order to cause significant inhibition. These studies suggested that although these drugs appear to specifically inhibit the activity of select lysosomal enzymes (i.e., ASMase), they do not directly inhibit them. The indirect inhibition of these enzymes has been hypothesized to occur through drug-induced degradation of the enzymes by blocking their intralysosomal binding and promoting their degradation by luminal proteases (Hurwitz, Ferlinz et al. 1994; Kolzer, Werth et al. 2004). Additionally, the similarity of the drug-induced lipidosis to Niemann-Pick disease has resulted in the hypothesis that these drugs actually inhibit the intracellular vesicle-mediated trafficking of lipids rather than their actual enzymatic metabolism (Underwood, Andemariam et al. 1996), as has been hypothesized to be the case in Niemann-Pick disease. Illustrations of the various hypothetical mechanisms of the drug-induced lipidosis are represented in Figure 1.19.

Although the biochemical mechanism through which drugs cause the gross accumulation of lipids is poorly understood, a variety of functional screening methods exists to assess this cytopathology in the drug development process. These models simply allow drugs to be studied for their ability to cause the phenotypic accumulation of lipids within the lysosomal compartment and have resulted in high throughput studies

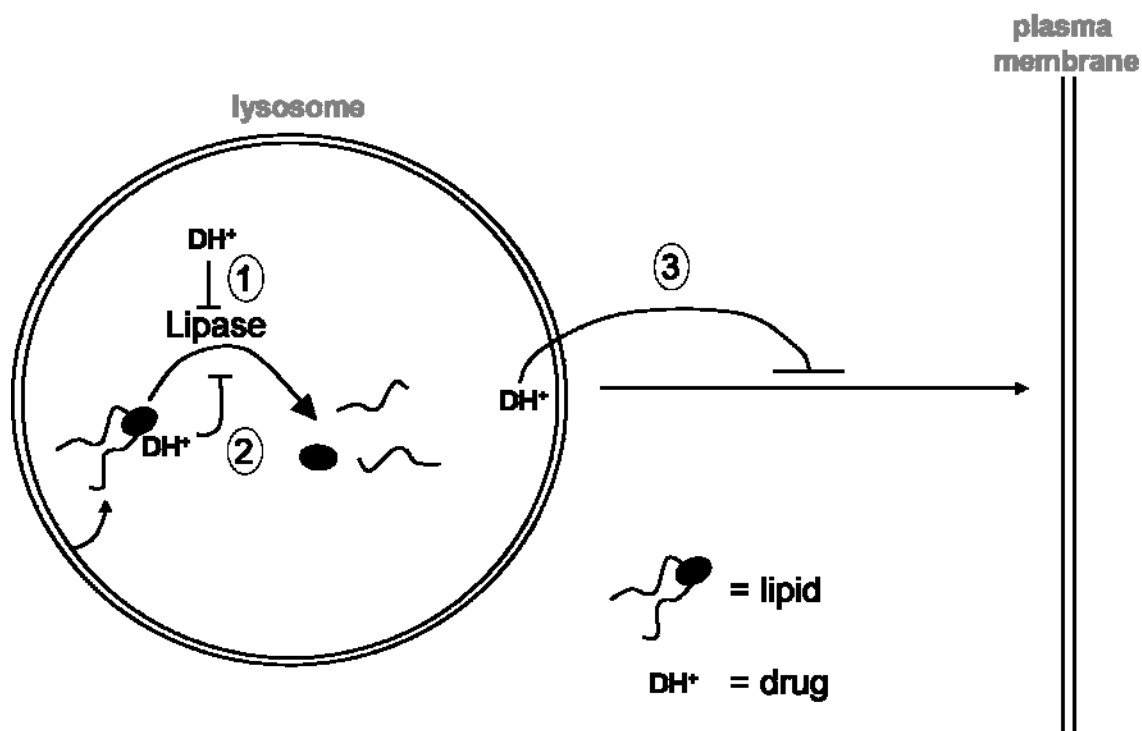


Figure 1.19. Amine-induced lysosomal lipidosis. Illustrated are the hypothesized mechanisms by which amine-drugs accumulating in the lysosome (DH^+) cause the lysosomal accumulation of lipids. At the membrane, lipids are metabolized by lipases to form degradation products. Mechanism 1 is the inhibition of lysosomal hydrolase activity, either directly or indirectly. Mechanism 2 is the formation of drug-lipid complexes that inhibit lipid metabolism. Mechanism 3 is the inhibition of vesicle-mediated trafficking of lipids out of the lysosome.

evaluating the drug properties that are critical and predictive for the drug-induced lipidosis. These studies have found the primary pharmacophore to consist of a molecule that is both weakly basic and hydrophobic. The diversity of pharmacological agents with vastly different primary mechanisms of actions that fall into this category suggests that the drug-induced lipidosis is not related to any distinct pharmacological class of drugs but rather a non-specific effect associated with the physicochemical properties of the drug. The physicochemical properties used to describe the weakly basic component are its pKa value and the calculated net charge of the drug at pH 4.0 (NC pH 4.0) (Tomizawa, Sugano et al. 2006). These drugs typically have pKa values around 8 to 10, but using the parameter of NC pH 4.0 allows for the exclusion of drugs that contain a weakly basic amine but are zwitterionic. The hydrophobicity of these drugs is best represented by logP, which is often predicted using quantitative structure property relationships (ClogP). Using data from a series of publications and experimental measurements of drug-induced lipidosis, by measuring the cellular accumulation of the fluorescent phospholipid analog, NBD-PE, a series of compounds was characterized as either inducers of the lipidosis (positive) or not (negative). A graph of ClogP versus NC pH 4.0 (Figure 1.20) for 63 compounds shows that compounds positive for the drug-induced lipidosis correlated with a ClogP > 1 and a NC pH 4.0 \geq 1 (Tomizawa, Sugano et al. 2006).

The cationic property of these drugs represented by pKa or NC pH 4.0 is thought to be of primary importance because this group imparts lysosomotropic properties to the drug and allows them to reach extremely high concentrations within the lysosome. The hydrophobic region on the other hand is thought to be important because it allows the

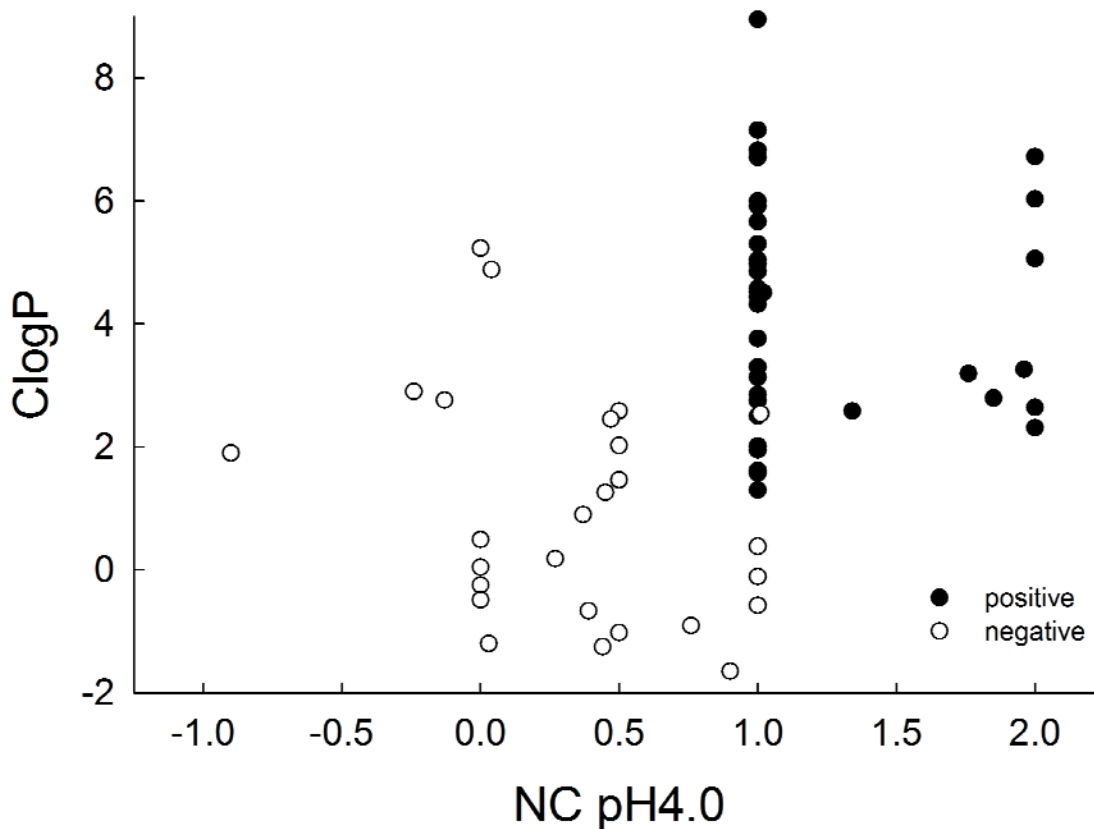


Figure 1.20. Physicochemical predictors of drug-induced lipidosis. Sixty-three drugs characterized as either positive (filled circles) or negative (open circles) in their ability to induce a lipidosis were plotted as a function of their physicochemical properties. Calculations of the physicochemical descriptors of hydrophobicity (ClogP) and net charge at pH 4.0 (NC pH4.0) for each drug allowed a plot of ClogP versus NC pH 4.0 for these drugs. Drugs that were positive for the lipidosis appeared to have a weakly basic, ionizable amine (NC pH4.0 ≥ 1) and to be hydrophobic (ClogP > 1). Reproduced with permission the Japanese Society of Toxicology (Tomizawa, Sugano et al. 2006).

drug to associate with intralysosomal targets, such as proteins and/or lipids. Because these compounds typically contain a hydrophilic region of the molecule (i.e., ionizable amine) along with a hydrophobic region, many of these molecules have surfactant properties that may play a role in their ability to cause the lipidosis (Schreier, Malheiros et al. 2000), possibly by associating with the interior lumen of the lysosomal membrane and altering its fluidity. Such changes in the properties of the membrane may impact the fusion and fission events necessary for the normal trafficking of lysosomes and their cargoes, or it may impact the activity of membrane-associated proteins resulting in defects in lysosomal lipid metabolism and/or trafficking.

Lysosomal ion trapping of hydrophobic amine-containing compounds that are commonly associated with the drug-induced lipidosis has been attributed to both the pharmacokinetic and pharmacodynamic properties of many of these agents. Specifically, the slow and significant accumulation of hydrophobic amine-containing psychotropic drugs in brain tissue has been associated with the anomalous therapeutic latency often observed with these agents (Kornhuber, Retz et al. 1995). Postmortem determination of amantadine levels in brain tissue showed that 8 days of treatment were necessary to reach 50% tissue saturation. This prolonged distribution phase was hypothesized to exist for other psychotropic drugs, such as antidepressants, and represents a novel hypothesis to the observed delay in therapeutic response with these agents. Therefore, the long distribution phase of these drugs may impact their time to onset of activity. In addition, the intralysosomal accumulation of lipids caused by the hydrophobic amine, U18666A, has been hypothesized to stabilize lysosomal membranes and prevent the lysosomal release of pro-apoptotic enzymes (Appelqvist,

Nilsson et al. 2011), as well as, suppress the production of HIV-1 in a cell culture model (Tang, Leao et al. 2009). Similarly, the hydrophobic amine, amiodarone, has been shown to reduce the infection of Vero cells with SARS coronavirus (Stadler, Ha et al. 2008) by some unknown mechanism possibly related to the lipidosis-inducing effect of this drug.

Of therapeutic interest, it has also been found that following the induction of a lipidosis in lung alveolar macrophages amiodarone, as well as its metabolite desethylamiodarone, accumulate over 10-fold that seen in non-lipidotic macrophages (Reasor 1991). These results suggest that the lipidosis itself may enhance the tissue accumulation of hydrophobic amine-drugs. This suggests that one hydrophobic amine-containing drug can influence the pharmacokinetic properties of a second by increasing its distribution to and accumulation in tissues. Such an effect may signify the potential for a distribution-based pharmacokinetic drug-drug interaction. Studies on single-versus multiple-dose pharmacokinetics have supported a similar effect whereby prolonged exposure to the hydrophobic amine, chlorphentermine, results in its increased tissue accumulation with chronic dosing (Lullmann, Rossen et al. 1973), suggesting the possibility that the lipidosis induced by the drug may facilitate an increase in its own apparent volume of distribution and half-life with chronic administration.

1.6.5. Importance of drug hydrophobicity

In this review, data from a variety of studies illustrating the ability of amine-containing compounds to alter lysosomal structure and function have been presented.

Collectively, the data have supported a general trend that the hydrophobicity of the drug is a key predictor in the potency and ability of the amine-containing drug to induce these different effects. An illustration representing the general trends shows the relationship between the hydrophobicity of the lysosomotropic amine-containing drug and its ability to cause lysosomal alkalization, cytoplasmic vacuolization and the lysosomal lipidosis (Figure 1.21). The potency of a compound in causing alkalization of lysosomes is directly related to the hydrophobicity of the drug. Similarly, the lysosomal lipidosis is shown to be directly related to drug hydrophobicity, but unlike lysosomal alkalization, drugs of sufficiently low hydrophobicity aren't capable of causing the lipidosis. In contrast, the cytoplasmic vacuolization is inversely related to drug hydrophobicity, with less hydrophobic compounds highly capable of causing the vacuolization, whereas the more hydrophobic compounds fail to elicit the effect.

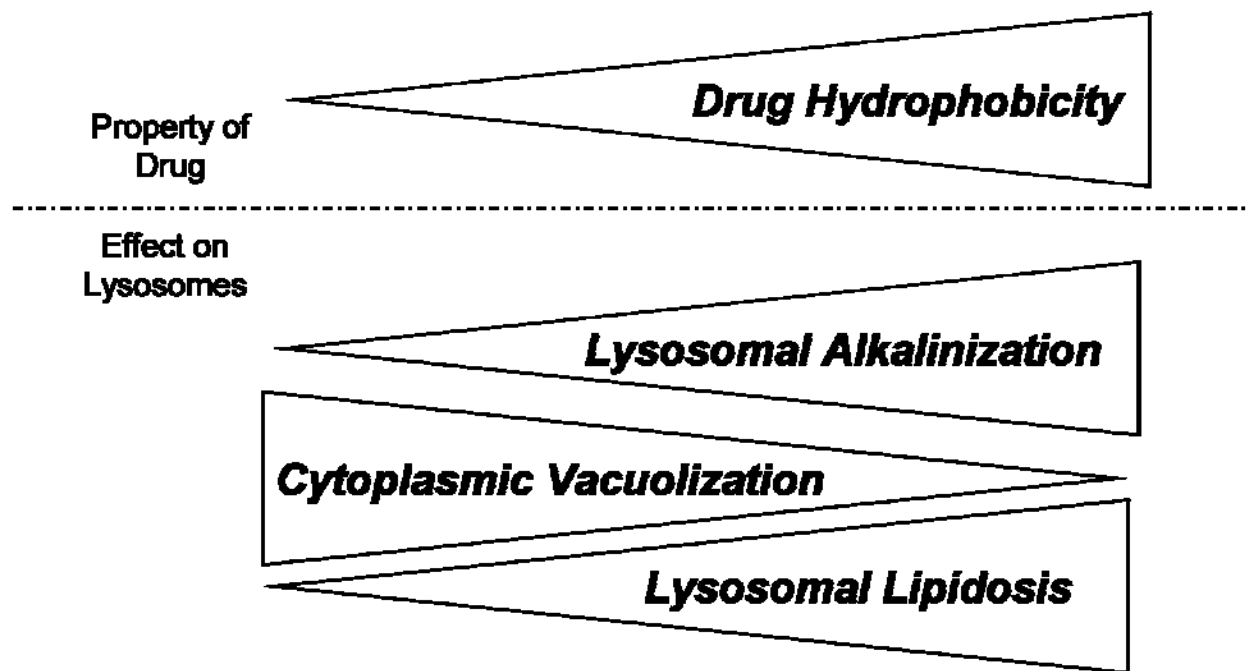


Figure 1.21. Empirical relationship between drug hydrophobicity and observed effect on lysosomes. The hydrophobicity of lysosomotropic amine-containing drugs appears to be related to the activity of the drug in causing different effects on lysosomes. Increased hydrophobicity is associated with lysosomal alkalinization and lysosomal lipidosis. Decreased hydrophobicity is associated with cytoplasmic vacuolization.

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Chapter 2

**Cationic amphiphilic drug treatment results in enhanced cellular
accumulation of substrates for ion trapping in lysosomes**

2.1. Introduction

In this chapter, the effect of cationic amphiphilic drug (CAD) treatment on the cellular accumulation and distribution of substrates for lysosomal ion trapping is evaluated. Cellular accumulation of the fluorescent substrate for lysosomal ion trapping, LysoTracker Red (LTR), is evaluated in CAD treated cells. The model CAD, imipramine, is found to cause an increase in the vesicular accumulation of LTR similar to that seen in NPC disease fibroblasts. Total cellular accumulation of LTR is increased in both CAD treated and Niemann-Pick type C (NPC) disease fibroblasts. Additional amine-containing compounds that are substrates for lysosomal ion trapping are increased in both vesicular staining and total cellular accumulation following CAD treatment. Amine-containing compounds that are not substrates for lysosomal ion trapping do not change in cellular distribution or accumulation following CAD treatment. These findings are consistent with the hypothesis that CADs cause a cellular lipidosis similar to that seen in NPC disease fibroblasts resulting in an increase in the lysosomal ion trapping capacity of cells. It is further hypothesized that the increase in lysosomal ion trapping capacity results from an expansion of the lysosomal compartment that is secondary to the lipidosis found in CAD treated and NPC disease cells.

Weakly basic amine-containing drugs accumulate in lysosomes (i.e., lysosomotropic amines) through an ion trapping-based mechanism that results in intralysosomal drug concentrations several orders of magnitude greater than that seen in the rest of the cell (Duvvuri and Krise 2005). In addition to the contribution of lysosomal ion trapping to the cellular uptake of these drugs, high intralysosomal drug concentrations can cause a number of structural and functional perturbations in the

lysosome. In particular, a group of hydrophobic amine-containing drugs known as CADs cause the lysosomal accumulation of lipids similar to that seen in diseases resulting from inborn errors in lysosomal lipid metabolism, such as NPC disease (Roff, Goldin et al. 1991). Interestingly, NPC disease cells have been shown to not only have enhanced accumulation of lipids but also enhanced cellular accumulation of substrates for lysosomal ion trapping (Kopitz, Gerhard et al. 1994; Kopitz, Harzer et al. 1996; Kaufmann and Krise 2008). The mechanism through which amine-containing compounds hyperaccumulate in NPC disease cells remains unknown but may be directly related to the function of the mutated protein or may be secondary to the disease-induced lipidosi. Nonetheless, the similarity between the NPC disease cells and CAD treated cells led to the hypothesis that CAD treatment would similarly increase the cellular accumulation of amine-containing compounds.

The ion trapping of weakly basic amine-containing compounds in lysosomes depends on the pH gradient existing between the acidic lumen of the lysosome (~pH 4-5) and the extralysosomal compartments (~pH 7.0). Amine-containing drugs entering the lysosome through passive diffusion experience a large shift in their ionic distribution, favoring ionization. Because of a marked decrease in the rate of passive diffusion of the ionized drug across membranes it becomes effectively trapped within the lysosome (de Duve, de Barsy et al. 1974). The accumulation of drug in lysosomes can account for a significant portion of the cellular accumulation of these drugs (Cramb 1986). Therefore, changes in the lysosomal properties that dictate cellular drug accumulation by lysosomal ion trapping can significantly affect the cellular accumulation of these drugs. For example, the cellular accumulation of these drugs depends on both the

volume and pH of the lysosomal compartment (de Duve, de Barsy et al. 1974), with changes in either resulting in alterations in the lysosomal ion trapping capacity of cells. Microscopy and cellular accumulation studies have suggested that NPC disease cells hyperaccumulate amine-containing compounds in an expanded aqueous lysosomal storage compartment (Kopitz, Harzer et al. 1996; Kaufmann and Krise 2008).

Although NPC disease is perhaps best known as a lysosomal storage disease resulting in the pronounced lysosomal accumulation of cholesterol (Lange, Ye et al. 1998) and other lipids (Walkley and Vanier 2009), it has also been shown to result in the lysosomal accumulation of amine-containing compounds. Development of early diagnostic tests actually measured the increased cellular accumulation of the lysosomotropic amine, methylamine, as a diagnostic marker for the disease (Kopitz, Gerhard et al. 1994). Later studies suggested that one of the proteins responsible for NPC disease, NPC1, functions as a molecular transporter in the movement of cargoes, including amine-containing compounds such as acriflavine (Davies, Chen et al. 2000). Additional studies have suggested that NPC1 may function in the vesicle-mediated trafficking of lysosomes and their amine-containing cargo (Kaufmann and Krise 2008). Therefore, lysosomal accumulation of amine-containing compounds in NPC disease cells may in fact result directly from dysfunctions in the proteins responsible for the disease or as hypothesized here it may be a secondary product of the expanded lysosomal compartment resulting from the lysosomal lipidosis.

CADs are generally described as molecules containing a hydrophobic ring system and a hydrophilic side chain, which together make the molecule amphiphilic. The hydrophilic side chain contains a weakly basic ionizable amine that is cationic in its

protonated form and imparts lysosomal accumulation by ion trapping. In addition, the ionizable amine and the relative hydrophobicity of the molecule have been postulated to facilitate intralysosomal binding to lipids and other membrane constituents (Halliwell 1997). CADs have been shown to cause the lysosomal accumulation of a variety of lipid species, including phospholipids (Lullmann, Lullmann-Rauch et al. 1973) and cholesterol (Yoshikawa 1991). Within the lysosome they have been hypothesized to directly and indirectly inhibit lysosomal lipase activity, form digestion resistant drug-lipid complexes (Anderson and Borlak 2006) and inhibit NPC1-dependent vesicle-mediated lipid trafficking out of the lysosome (Lange, Ye et al. 2000). Whatever the biochemical mechanism for the lipidoses, its similarity to that seen in NPC disease cells has resulted in the use of CADs to chemically induce NPC disease in cell culture models and led to the hypothesis that CADs will also cause an increase in the cellular accumulation of substrates for lysosomal ion trapping.

2.2. Materials and methods

2.2.1. Cell lines and reagents

LysoTracker Red DND-99 (LTR), LysoTracker Green DND-26, MitoTracker Red FM, sulforhodamine 101, Dulbecco's phosphate buffered saline (D-PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Daunorubicin was purchased from Oakwood Products (West Columbia, SC). Propranolol and imipramine were purchased from Sigma-Aldrich (St. Louis, MO). ³H-propranolol was purchased from GE Healthcare (Waukesha, WI). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

Wildtype (WT) human fibroblasts (catalogue # CRL-2076) and purchased from ATCC (Manassas, VA). NPC1^{-/-} (catalogue # GM03123) and NPC2^{-/-} (catalogue # GM18455) human fibroblasts were purchased from Coriell Cell Repository (Camden, NJ). Cells were maintained in DMEM supplemented with 10% FBS and maintained at 37°C and 5% CO₂, all experiments were carried out under these conditions unless otherwise stated. Cells were routinely subcultured to maintain 60% to 80% confluency.

2.2.2. Cellular amine accumulation assays

Fibroblasts were seeded in multi-well polystyrene tissue culture plates and allowed to adhere overnight. Cells were treated 10 µM imipramine or vehicle alone for 48 hours under normal growth conditions. In the last 2 hours, the amine-containing probes were added. Concentrations of 100 nM LTR, 1 µM LysoTracker Green, 1 µM daunorubicin, 5 µM sulforhodamine 101, 100 nM MitoTracker Red and 100 nM propranolol containing 10 nM ³H-propranolol were used. After the 2-hour exposure under normal growth conditions cells were washed twice with D-PBS rapidly to prevent loss of cell-associated probe by diffusion. Cells were lysed in either 0.1 M NaOH or lysis buffer containing 50 mM tris base, 150 mM NaCl, 1% NP40 adjusted to pH 7.4. Lysed samples were immediately assessed for fluorescence or radioactivity. Fluorescence was measured using a Bio-Tek FL600 microplate fluorescence reader equipped with the appropriate excitation and emission filters for each fluorophore. Radioactivity was determined by adding the lysate sample to ScintiVerse BD Cocktail (Fisher) with radioactivity in DPM measured by a Beckman LS 60001C liquid scintillation counter. Background signal contribution from non-specific binding of

compound to polystyrene surfaces or from the cells alone was subtracted from the sample measurements. Cell protein content was measured using the BCA method. Fluorescence (RFU) or radioactivity (DPM) counts were normalized to cellular protein and overall cellular accumulation of the probe was expressed as a function of cellular protein in micrograms.

2.2.3. Cellular microscopy and probe distribution

Fluorescence microscopy of amine-containing probe distribution in fibroblasts treated with 10 μ M imipramine or vehicle alone were carried out using the same treatment regimen as done in the 'Cellular amine accumulation assays' except cells were grown on glass coverslips. Following the 48-hour imipramine treatment and 2-hour exposure to the amine-containing fluorophores, the cells were washed twice with D-PBS and the cells on coverslips were immediately mounted on microscope slides for fluorescence imaging. The cells were viewed using a Nikon Eclipse 80i microscope equipped with a 40x (1.30 NA) oil immersion objective. Fluorescence images were acquired using the filter cube that best overlapped with the documented spectral properties of each fluorophore. Samples for fluorescence imaging were excited with an EXFO X-Cite 120 PC fluorescence illumination system. Images were acquired using an ORCA ER camera (Hamamatsu). Images were analyzed using Metamorph version 7.0 (Universal Imaging) or ImageJ (free online at rsbweb.nih.gov) software. Background images of cells not exposed to fluorescent probe were acquired to correct for autofluorescence and images were scaled identically to allow for comparison.

2.3. Results and discussion

2.3.1. Intracellular distribution and cellular accumulation of amine-containing compounds in CAD treated and NPC disease fibroblasts

The initial studies sought to compare NPC disease cells to CAD treated cells in their capacity to accumulate an amine-containing substrate for lysosomal ion trapping. Therefore, the fluorescent substrate for lysosomal ion trapping, LTR, was identified as a model compound that could be monitored by fluorescence microscopy to qualitatively assess the cellular accumulation and distribution of the compound. In addition, its fluorescence properties facilitate quantitative measurements of cellular accumulation of the probe. The model CAD chosen for these studies was imipramine because of its well-documented ability to cause a cellular lipidosis similar to that seen in NPC disease cells (Rodriguez-Lafrasse, Rousson et al. 1990). Cell lines used included WT fibroblasts and NPC disease fibroblasts that result from mutations in the late endosomal/lysosomal proteins Niemann-Pick C1 (NPC1) or Niemann-Pick C2 (NPC2), denoted NPC1^{-/-} and NPC2^{-/-}, respectively. Mutations in either protein, NPC1 or NPC2, result in a nearly identical phenotype that is described by the lysosomal accumulation of cholesterol and other lipids (Chang, Reid et al. 2005) and has been related to a general reduction of egress of cholesterol and other cargoes out of the lysosome (Neufeld, Wastney et al. 1999; Kaufmann and Krise 2008; Goldman and Krise 2010).

Fluorescence microscopy studies of LTR accumulation and distribution were carried out in WT fibroblasts, with or without imipramine treatment, and NPC1^{-/-} fibroblasts (Figure 2.1). Similar to previous studies, NPC disease fibroblasts show a

LysoTracker Red

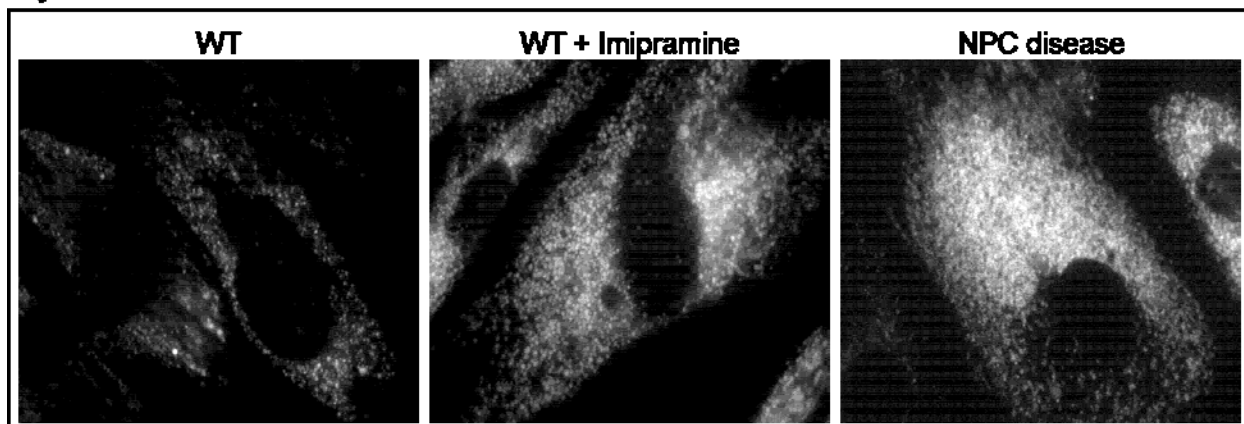


Figure 2.1. Increased vesicular accumulation of LTR in CAD treated and NPC disease fibroblasts. Wildtype (WT) control fibroblasts were left untreated or treated with 10 μ M imipramine (WT + imipramine) for 24 hours under normal growth conditions. WT and imipramine treated WT fibroblasts along with NPC disease (NPC1^{-/-}) fibroblasts were exposed to 200 nM LTR for 2 hours prior to imaging. Compared to WT cells, both imipramine treated WT cells and NPC disease cells show an increase in the vesicular accumulation of the fluorescent substrate for lysosomal ion trapping, LTR.

significant increase in the punctate vesicular staining of LTR as compared to WT fibroblasts (Kaufmann and Krise 2008), suggesting an increase in the cellular accumulation of LTR in an expanded lysosomal compartment. Similarly, WT fibroblasts exposed to the lipidosi-inducing imipramine treatment also resulted in a significant increase in the punctate vesicular accumulation of LTR. These results suggest that CADs cause the accumulation of substrates for lysosomal ion trapping, similar to that observed in NPC disease cells through an expansion of the lysosomal compartment.

To quantitatively verify the increased cellular accumulation of LTR observed by fluorescence microscopy, the total cellular accumulation of LTR was measured in WT fibroblasts with or without imipramine treatment, as well as, in NPC1^{-/-} and NPC2^{-/-} fibroblasts (Figure 2.2). In agreement with the fluorescence microscopy results, LTR accumulated to a much higher degree in both NPC disease cell lines (i.e., NPC1^{-/-} and NPC2^{-/-}) as compared to WT cells. Similarly, imipramine treatment significantly increased LTR accumulation in WT cells confirming the results from the microscopy studies. Together these results suggest that both the NPC disease-induced lipidosi and the CAD-induced lipidosi result in an increase in the ability of cells to accumulate weakly basic amine-containing compounds as illustrated by both the microscopy and cellular uptake studies. In addition, the microscopy studies suggest that the increase in cellular accumulation of these amine-containing compounds occurs through increased accumulation in vesicular compartments presumed to be lysosomes. Since LTR and similar compounds accumulate in lysosomes through an ion trapping-based mechanism, it was further hypothesized that the lipidosi results in an increase in the

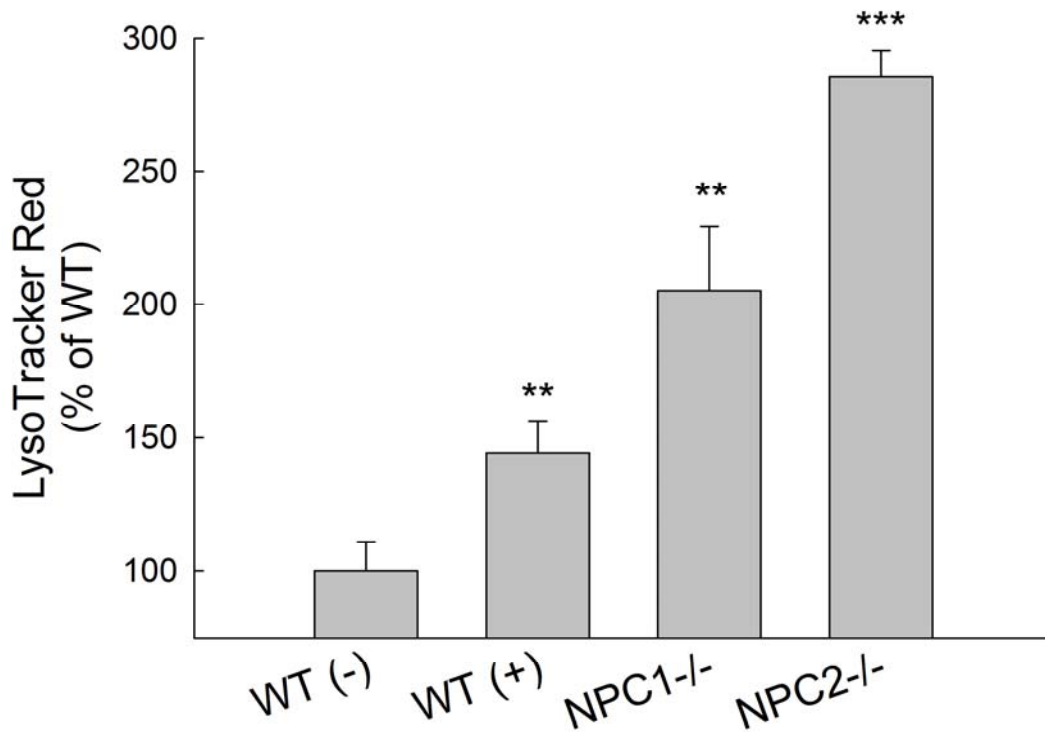


Figure 2.2. CAD treatment increases the cellular accumulation of LTR, similar to NPC disease cells. WT human fibroblasts were treated with 10 μ M imipramine (WT (+)) or vehicle alone (WT (-)) for 24 hours. In the last 2 hours of treatment, 200 nM LTR was added to the WT fibroblasts or NPC disease fibroblasts with mutations in either NPC1 (NPC1^{-/-}) or NPC2 (NPC2^{-/-}). At the end of the treatment period cells were lysed and assessed for total cellular accumulation of LTR. NPC disease cells accumulate more LTR than WT cells. Imipramine treatment increased the cellular accumulation of LTR in WT fibroblasts. Cellular accumulation of LTR was assessed by measuring fluorescence signal in the cell lysate and normalized to cellular protein content. Cellular accumulation of LTR was normalized to the vehicle-treated WT cells and expressed as a percentage of control WT. Cellular accumulation of LTR is represented as mean \pm S.D. of at least three values for each sample (**, $p < 0.01$; ***, $p < 0.001$ by Student's t test).

lysosomal ion trapping capacity of cells through an expansion of the lysosomal compartment.

2.3.2. Intracellular distribution and cellular accumulation of amine-containing compounds following CAD treatment

Although CAD treatment was found to increase the cellular accumulation of an amine-containing substrate for lysosomal ion trapping, this observation does not necessarily establish that the lysosomal ion trapping capacity of the cell is increased. In addition to the lipidoses, CADs are surface-active agents that have been suggested to increase the cellular accumulation of drugs by enhancing the permeability of the plasma membrane (Tsao, Iga et al. 1982; Drori, Eytan et al. 1995). Perhaps another mechanism of increased cellular accumulation of the amine-containing compounds would be through an increase in cellular cationic binding sites. To further identify the mechanism through which the CAD treatment increases the cellular accumulation of amine-containing compounds a series of amine-containing compounds that can be classified as lysosomotropic or non-lysosomotropic were identified. These compounds and their structures are illustrated in Figure 2.3. The lysosomotropic compounds are all well-established substrates for lysosomal ion trapping and includes: daunorubicin (Gong, Duvvuri et al. 2003), LysoTracker Red (Lemieux, Percival et al. 2004), LysoTracker Green (Zheng, Zhang et al. 2010) and propranolol (Cramb 1986). The non-lysosomotropic compounds on the other hand accumulate in cells through non-ion trapping mediated mechanisms. Sulforhodamine 101 is a zwitterionic compound

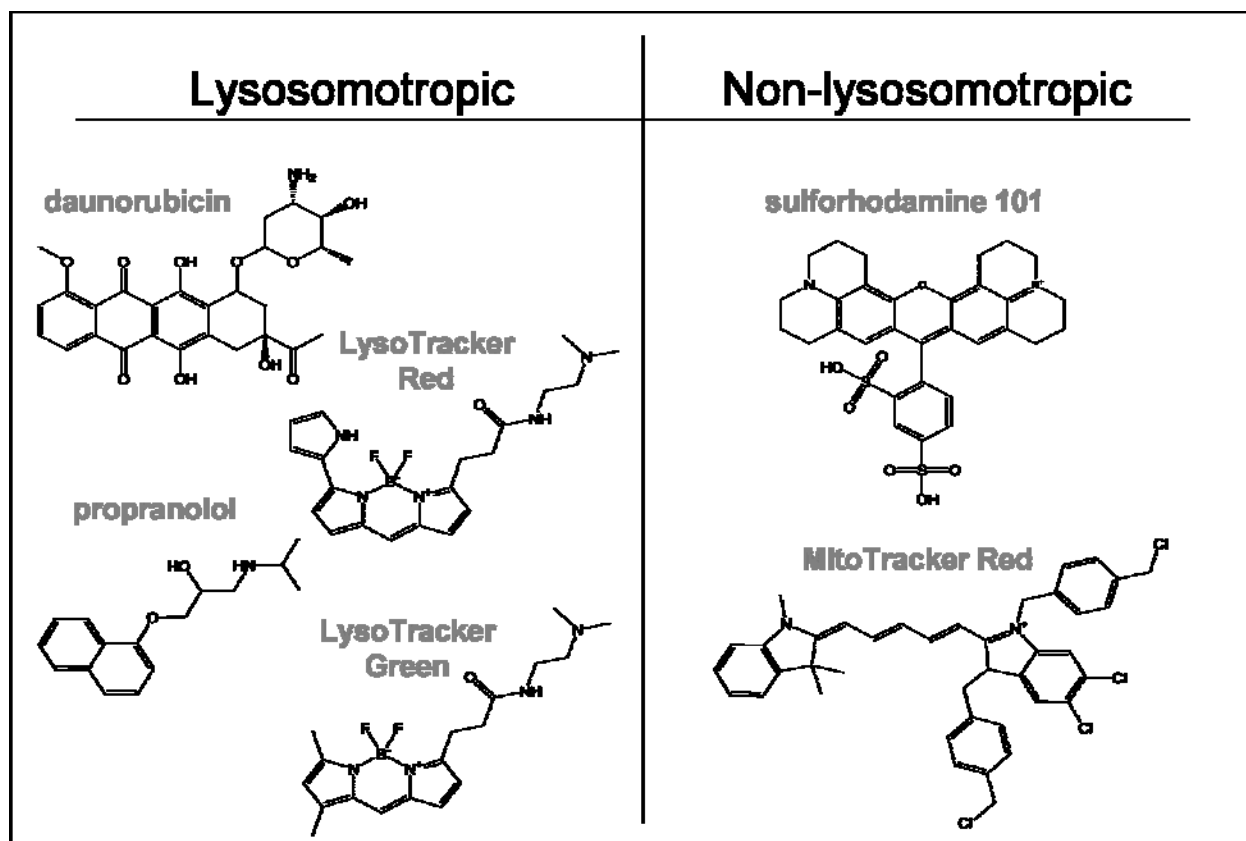


Figure 2.3. Model amine-containing compounds characterized as lysosomotropic or non-lysosomotropic. Lysosomotropic amine-containing compounds are substrates for extensive cellular accumulation by ion trapping in lysosomes, whereas non-lysosomotropic compounds are amine-containing compounds but fail to accumulate in cells by ion trapping in lysosomes. Cellular accumulation of these compounds was measured following a lipodosis-inducing treatment with imipramine. When possible fluorescence microscopy was used to assess the intracellular distribution of these compounds.

thought to accumulate in cells through fluid-phase endocytosis (Colin, Limagne et al. 2011) and has been shown to be a substrate for the multidrug resistance-related protein (MRP1), but its uptake is insensitive to intracellular pH gradients and is therefore presumed not to be a substrate for lysosomal ion trapping (Gong, Duvvuri et al. 2003). MitoTracker Red is a cationic fluorophore that accumulates in, and specifically labels, mitochondria based on the negative membrane potential of the organelle (Poot, Zhang et al. 1996). If only the lysosomotropic amines are affected by CAD treatment, then an increase in lysosomal ion trapping capacity would be suggested. If the non-lysosomotropic amines are also hyperaccumulated, then an increase in cationic binding sites or an increase in the membrane permeability will be suggested.

WT fibroblasts were treated with the CAD, imipramine, at the lipidosis-inducing concentration of 10 μ M for 48 hours and the cellular accumulation and distribution of the lysosomotropic and non-lysosomotropic compounds were assessed following a 2 hour exposure. Fluorescence imaging revealed that imipramine causes a significant increase in the vesicular accumulation of the lysosomotropic amine-containing compounds, including LTR, LysoTracker Green and daunorubicin (Figure 2.4). Imaging of sulforhodamine 101 and MitoTracker Red revealed no change in the cellular accumulation or distribution of these compounds following imipramine treatment (Figure 2.5).

Similarly, imipramine treatment resulted in an increase in the cellular accumulation of the lysosomotropic amines, including LTR, LysoTracker Green, daunorubicin and propranolol (Figure 2.6). Cellular accumulation of sulforhodamine 101 and MitoTracker Red were found to be unchanged (Figure 2.7). Together these results

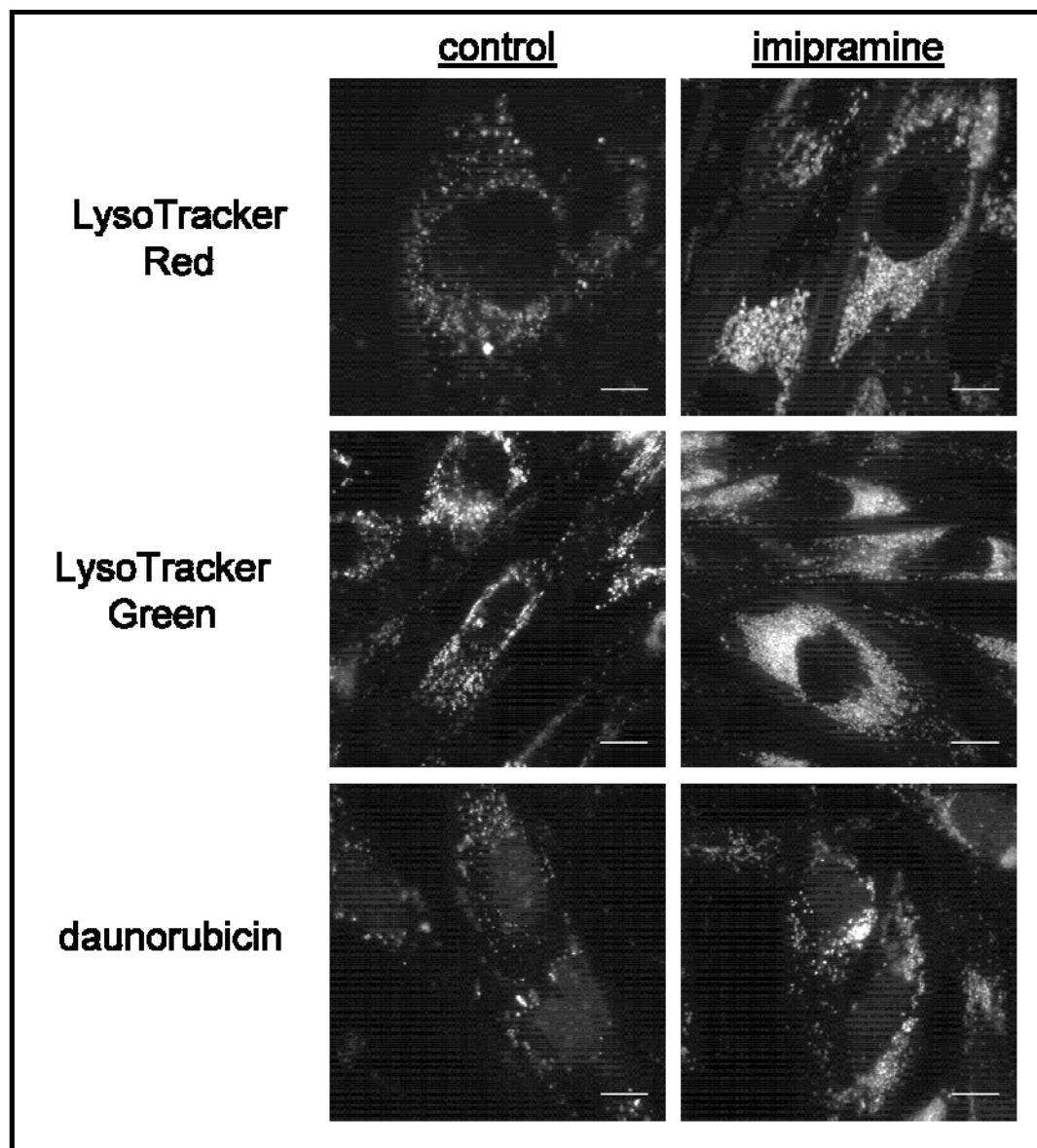


Figure 2.4. Increased vesicular accumulation of lysosomotropic amine-containing compounds following imipramine treatment. WT fibroblasts were left untreated (control) or treated with 10 μ M imipramine for 48 hours. 100 nM LysoTracker Red, 1 μ M LysoTracker Green or 1 μ M daunorubicin was added in the last 2 hours and cells were subsequently imaged by fluorescence microscopy. The lipodosis-inducing imipramine treatment increased the vesicular accumulation of all three lysosomotropic compounds.

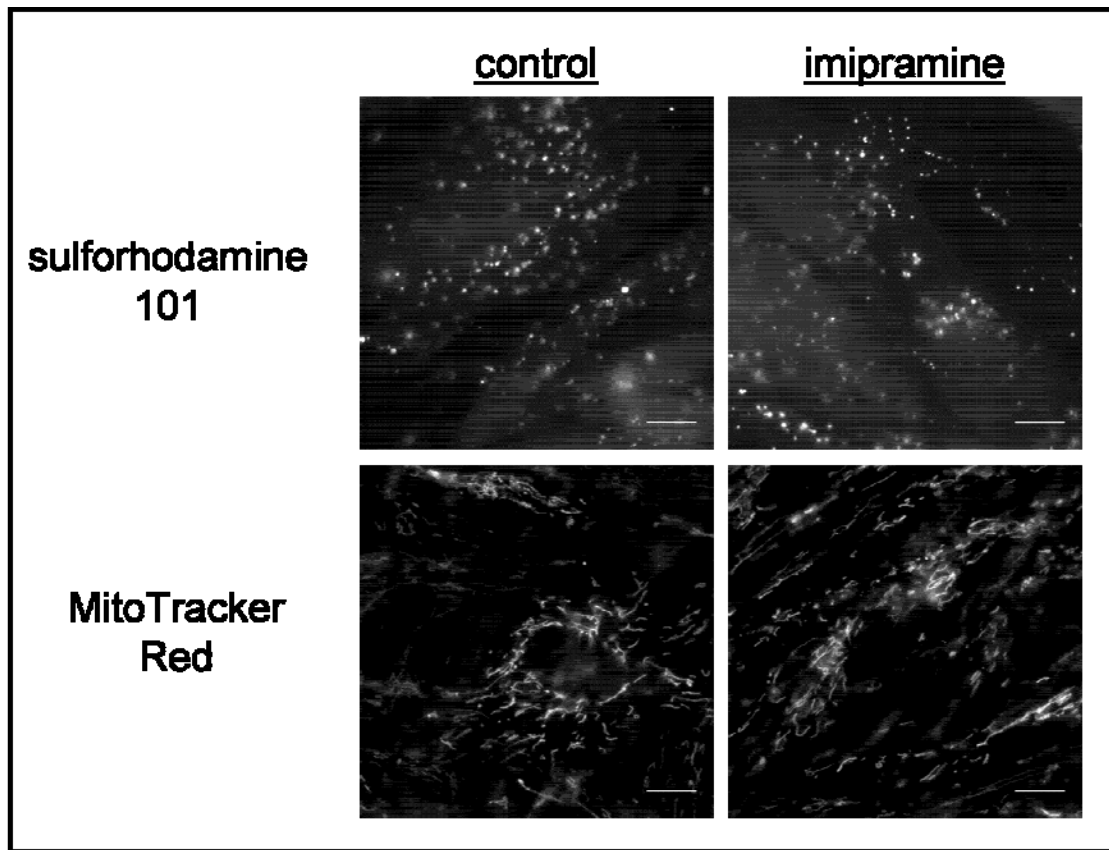


Figure 2.5. Imipramine treatment does not affect the cellular distribution or accumulation of non-lysosomotropic amine-containing compounds. WT fibroblasts were left untreated (control) or treated with 10 μ M imipramine for 48 hours. 100 nM MitoTracker Red or 5 μ M sulforhodamine 101 was added in the last 2 hours and cells were subsequently imaged by fluorescence microscopy. The lipodosis-inducing imipramine treatment had no effect on the cellular distribution or accumulation of either of the non-lysosomotropic compounds.

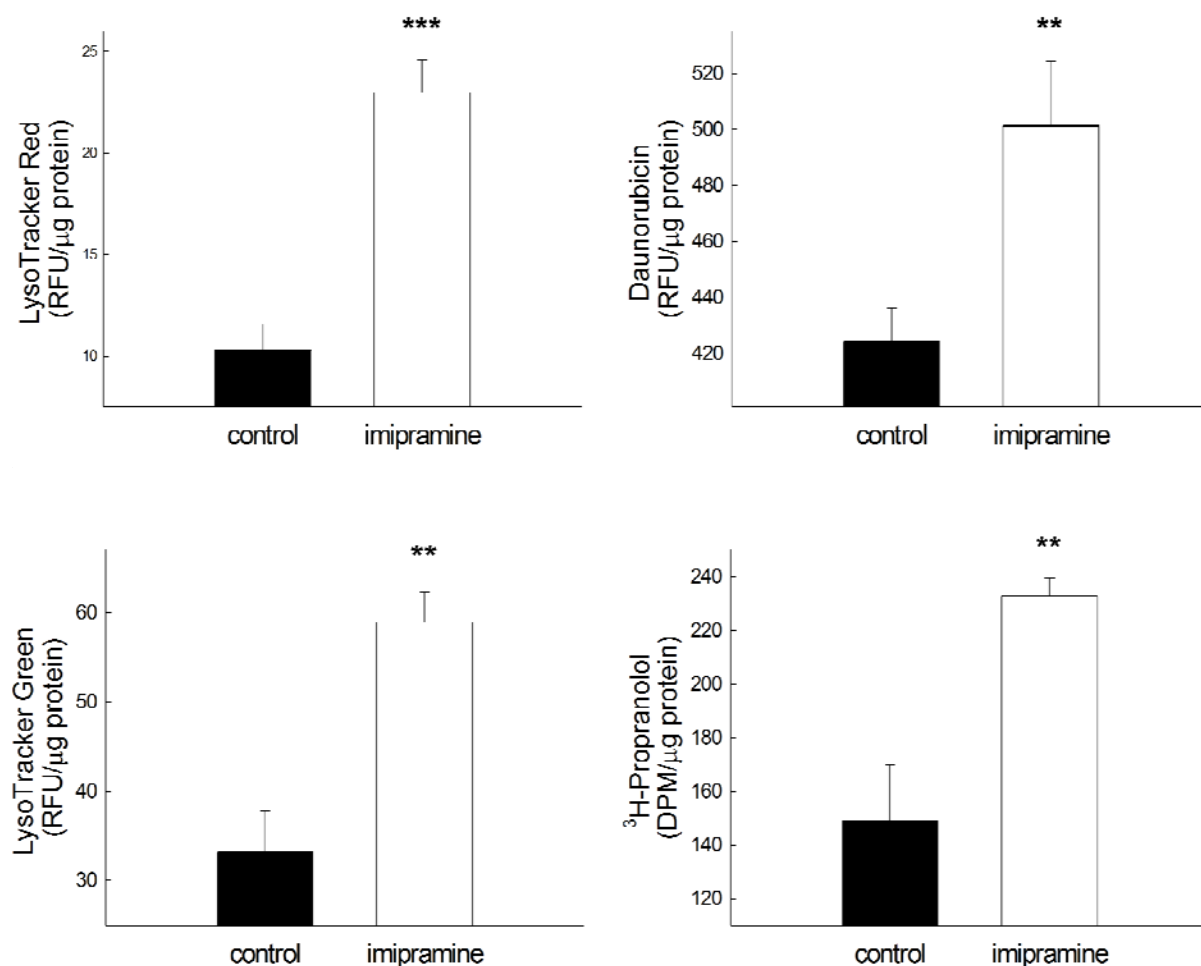


Figure 2.6. Increased cellular accumulation of lysosomotropic amine-containing compounds following imipramine treatment. WT fibroblasts were left untreated (control) or treated with 10 μ M imipramine for 48 hours. 100 nM LysoTracker Red, 1 μ M LysoTracker Green or 1 μ M daunorubicin, or 100 nM propranolol containing 10 nM 3 H-propranolol was added in the last 2 hours and cells were subsequently lysed and assessed for total cellular fluorescence or radioactivity. Cellular probe accumulation was normalized to cellular protein content and expressed as either RFU or DPM per microgram of protein. The lipodosis-inducing imipramine treatment increased the cellular accumulation of all four lysosomotropic compounds. Cellular accumulation is represented as mean \pm S.D. of at least three values for each sample (**, $p < 0.01$; ***, $p < 0.001$ by Student's t test).

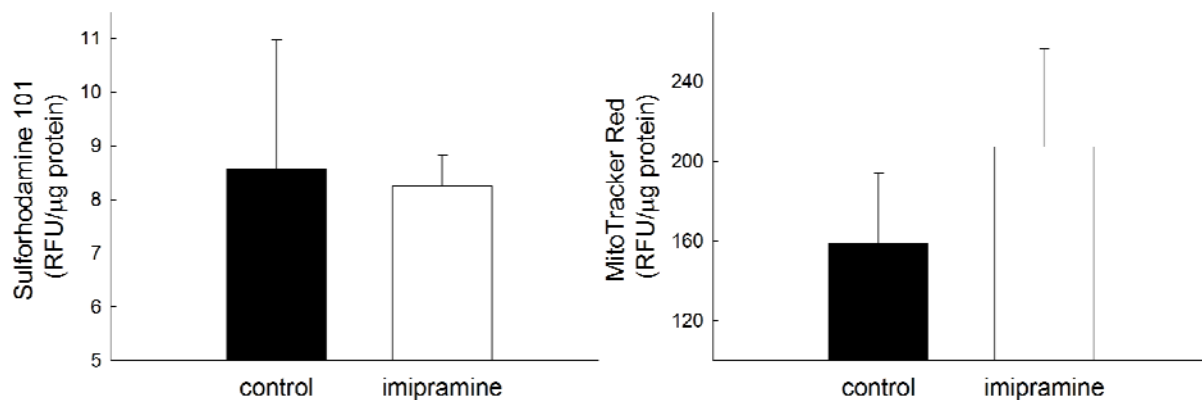


Figure 2.7. Imipramine treatment does not affect the cellular accumulation of non-lysosomotropic amine-containing compounds. WT fibroblasts were left untreated (control) or treated with 10 μ M imipramine for 48 hours. In the last 2 hours, 100 nM MitoTracker Red or 5 μ M sulforhodamine 101 was added and cells were subsequently lysed and assessed for total cellular fluorescence and normalized to cellular protein content. The lipidosis-inducing imipramine treatment did not affect the cellular accumulation of the non-lysosomotropic compounds. Cellular accumulation is represented as mean \pm S.D. of at least three values for each sample.

suggest that treatment with the CAD, imipramine, causes an increase in the cellular accumulation of substrates for lysosomal ion trapping without influencing the accumulation of amine-containing compounds that are not substrates for lysosomal ion trapping. The failure to affect non-lysosomotropic amines suggests that the increased accumulation is not due to a non-specific increase in cellular membrane permeability or an increase in intracellular binding sites for cationic drugs. Therefore, these results are in agreement with the hypothesis that CAD treatment causes an expansion of the lysosomal compartment resulting in an increased capacity of cells to accumulate substrates for ion trapping in lysosomes.

Although the mechanism for the increased accumulation of the lysosomotropic amine-containing drugs could result through several possible mechanisms here it is hypothesized that CADs cause an expansion of the lysosomal compartment. The observed increase in vesicular staining of the various lysosomotropic amines following imipramine treatment is consistent with this hypothesis. The observation that both the CAD treated and the NPC disease cells show a similar increase in vesicular staining further suggests that the lysosomal volume expansion is possibly secondary to the lysosomal lipidosis seen under these conditions. However, these results do not exclude the possibility that imipramine directly inhibits the NPC1-dependent transport of amines, especially since NPC1 has been hypothesized to function in the regulation of intracellular amine transport (Davies, Chen et al. 2000; Kaufmann and Krise 2008).

Regardless of the mechanism through which imipramine causes the increased cellular accumulation of lysosomotropic amine-containing compounds, the ability of one drug (i.e., imipramine) to increase the cellular accumulation of a second (i.e., LTR,

LysoTracker Green, daunorubicin, propranolol) is hypothesized to illustrate a potential distribution-based drug-drug interaction pathway. The 27% to 123% increase in the cellular accumulation of lysosomotropic amine-containing drugs following imipramine treatment suggests that, in vivo, imipramine could have a large impact on the tissue accumulation and distribution of these drugs. Increased tissue accumulation would be expected to result in an increase in the volume of distribution of these drugs and the increased distribution to lysosome-rich tissue would result in increased drug levels in tissues such as the liver and lung. Together this data suggests the CADs can cause an increase in the lysosomal ion trapping capacity of cells with increased cellular accumulation of weakly basic amine-containing drugs in an expanded lysosomal compartment that may have therapeutic implications, such as in drug-drug interactions. Further in vivo studies to illustrate its possible therapeutic relevance, as well as, in-depth in vitro studies to understand the molecular mechanism for this interaction are needed.

2.4. Conclusions

This work sought to illustrate the ability of a lipidosis-inducing CAD treatment to cause an increase in the cellular accumulation of substrates for lysosomal ion trapping by causing an expansion of the lysosomal compartment. The basis for this hypothesis was derived from the observation that cells from patients with the lysosomal lipid storage disease, NPC disease, hyperaccumulate amine-containing compounds in what appears to be an expanded lysosomal compartment (Kopitz, Harzer et al. 1996). Because CADs are well known for their ability to cause the lysosomal accumulation of

lipids, similar to that observed in NPC disease, it was hypothesized that CADs would similarly cause an increase in the cellular accumulation of amine-containing compounds that accumulate in lysosomes. Therefore, the initial studies sought to illustrate the ability of both NPC disease cells and cells treated with the CAD, imipramine, to show an increase in the vesicular staining and cellular accumulation of the substrate for lysosomal ion trapping, LTR. Imipramine was found to cause an increase in both the vesicular staining and cellular accumulation of LTR, similar to that observed in NPC disease cells. Subsequent studies measuring the cellular accumulation and distribution of lysosomotropic amines and non-lysosomotropic amines were consistent with a generalized increase in the cellular accumulation of substrates for lysosomal ion trapping following CAD treatment. Together, these results support a CAD-induced expansion of the lysosomal compartment that results in the enhanced capacity of cells to accumulate drugs through ion trapping-based accumulation in the expanded lysosomal compartment.

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Chapter 3

Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: Implications for a novel intracellular distribution-based drug-drug interaction

3.1. Introduction

The purpose of this chapter is to evaluate the ability of a class of drugs, known as CADs, to cause an increase in the cellular accumulation of compounds that are substrates for ion trapping-based accumulation in lysosomes and to characterize the mechanism of this intracellular distribution-based drug-drug interaction. As previously described in Chapter 1, lysosomes are a significant site for the cellular and tissue accumulation of weakly basic amine-containing drugs and by reaching concentrations within the lysosome several orders of magnitude greater than that seen in the extracellular space, these agents are capable of causing various physiological and morphological perturbations of the lysosomal apparatus. As described in Chapter 2, CADs are well-documented as causing the lysosomal accumulation of lipids (Halliwell 1997), similar to that seen in Niemann-Pick type C (NPC) disease (Sakuragawa, Sakuragaw et al. 1977; Roff, Goldin et al. 1991). Interestingly, the studies in Chapter 2 revealed that NPC disease cells and cells treated with the model CAD, imipramine, had an enhanced capacity to accumulate lysosomotropic amine compounds in perinuclear vesicular structures presumed to be lysosomes and suggested a relationship between the cellular lipidosis and the increased cellular accumulation of weakly basic amine-containing compounds.

Consistent with the findings in Chapter 2, *in vivo* studies have shown that following a drug-induced lipidosis, tissues hyperaccumulate amine-containing drugs when administered secondarily (Reasor 1991; Zheng, Zhang et al. 2010). In these studies, the increased accumulation of amine-containing drugs was proposed to occur through increased lipid-binding sites for the drugs. Here, the hypothesis is that CADs,

rather than increasing the intracellular binding sites for amine-drugs, actually increase the cellular accumulation of amine-containing drugs through an expansion of the aqueous volume of the lysosomal compartment; therefore, they increase the capacity of cells to accumulate drugs by lysosomal ion trapping. Further, it is hypothesized that the expansion of the lysosomal compartment is secondary to the drug-induced lipidosis. Such an ability of CADs to alter the cellular retention of other amine-containing drugs illustrates a novel distribution-based drug-drug interaction pathway that occurs at the intracellular level.

Previously described drug-drug interactions result from the ability of one drug to alter the pharmacokinetic properties of a second drug, including the absorption, distribution, metabolism, excretion or transport (ADMET) properties. The ability of these interactions to cause clinically relevant changes in drug efficacy and/or toxicity has encouraged researchers to elucidate the various pathways through which drugs can interact within the complex biological matrix that is the human body. Although the majority of this work involves drug interactions occurring through modulation of drug metabolizing enzymes (Ogu and Maxa 2000) or membrane drug transporters (Giacomini 1997), there has been an effort to describe how the propensity of amine-containing drugs to accumulate in lysosomes can result in distribution-based drug-drug interactions (Daniel 2003). Because of their tendency to accumulate in lysosomes and in lysosome-rich tissue (Daniel and Wojcikowski 1999) amine-containing drugs often have a high apparent volume of distribution (Bickel, Graber et al. 1983). Therefore, if factors, such as drug co-administration, can influence the deposition of these amine-containing drugs into lysosome-rich tissue then the distribution profile of these drugs

could be significantly altered. Such deviations in the distribution profile of the drugs are expected to have implications in drug efficacy and/or toxicity.

In Chapter 2 the model CAD, imipramine, was found to specifically increase the cellular accumulation of amine-containing compounds that are substrates for ion trapping-based accumulation in lysosomes. Here it is shown that exposure to a variety of drug molecules, which are classified as CADs, results in an increase in the cellular accumulation of secondarily administered amine-containing compounds. Further mechanistic studies reveal that imipramine causes an expansion of the lysosomal compartment of cells. The lysosomal volume expansion is found to occur in a time- and temperature-dependent manner, consistent with an energy-dependent cellular remodeling process. Treatments known to reverse the CAD-induced lipidosis prevent the apparent increase in lysosomal volume suggesting the volume expansion is secondary to the drug-induced lipidosis. The results here are consistent with the hypothesis that CADs, through their ability to inhibit cellular lipid metabolism, cause an increase in the lysosomal volume of cells resulting in the increased cellular uptake of lysosomotropic amine compounds, thus revealing a novel mechanism for intracellular distribution-based pharmacokinetic drug-drug interactions.

3.2. Materials and methods

3.2.1. Cell lines and reagents

LysoTracker Red DND-99 (LTR), LysoTracker Green DND-26 (LTG), anionic 70,000 mol. wt. Oregon Green Dextran, Dulbecco's phosphate buffered saline (D-PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen

(Carlsbad, CA). Daunorubicin was purchased from Oakwood Products (West Columbia, SC). Nigericin, monensin, hydroxypropyl- β -cyclodextrin (HPCD), propranolol, imipramine, concanamycin A, ammonium chloride, haloperidol, risperidone, chlorpromazine, lidocaine, bupivacaine, amiodarone, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). ^3H -propranolol was purchased from GE Healthcare (Waukesha, WI). ^{14}C -methylamine was purchased from Moravek Biochemicals (Brea, CA). Lipoprotein depleted serum (LPDS) was purchased from Millipore (Billerica, MA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). CRL-2076 normal human foreskin fibroblasts (WT) were obtained from ATCC (Manassas, VA). NPC1 $^{-/-}$ (catalogue # GM03123) and NPC2 $^{-/-}$ (catalogue # GM18455) human fibroblasts were purchased from Coriell Cell Repository (Camden, NJ). MDA-1986 squamous cell carcinoma cells were a generous gift from the laboratory of Dr. M.S. Cohen at the University of Kansas Medical Center. All cells were maintained in DMEM supplemented with 10% FBS and maintained at 37°C and 5% CO₂, all experiments were carried out under these conditions unless otherwise stated. Cells were routinely subcultured to maintain 60% to 80% confluency.

3.2.2. Drug treatments

Cells were seeded onto multi-well polystyrene plates and allowed to attach overnight. Imipramine and the other CADs were added at a concentration of 10 μM for 24 hours to 48 hours, unless otherwise specified. Lysosomal alkalinizing treatments, including; 30 μM chloroquine, 10 mM ammonium chloride and 200 nM concanamycin A were applied for a total drug treatment time of up to 2 hours. Lysosomal pH knockdown

experiments were conducted using a 2 hour treatment with the ionophores, 10 μ M nigericin and 20 μ M monensin. In pH dependent binding studies ionophores were added in pH adjusted buffer for 30 minutes to 2 hours just prior to the addition of the fluorescent or radioactive amine probe. The pH 5.0 and 6.0 buffers contained 150 mM sodium chloride, 20 mM Mes, 5 mM potassium chloride and 1 mM magnesium sulfate. The pH 7.3 buffer was similar except it contained 20 mM Hepes instead of Mes. In the temperature-dependency study, cells were either maintained in 37°C media or switched to 4°C media followed by the addition of imipramine or vehicle alone. After 4 hours at either 4°C or 37°C, cells were washed three times with 37°C D-PBS and replaced with fresh 37°C growth media without imipramine. In lipidosis-reversing treatments growth media was removed and replaced with either fresh growth media, fresh growth media supplemented with 0.1% HPCD or growth media without FBS but supplemented with 3 mg/ml LPDS. After 24 hours, either imipramine or vehicle alone was added to each treatment group for an additional 24 hours. In all experiments control cells were treated with vehicle only to control for potential vehicle effects.

3.2.3. Cell imaging studies

Cells were viewed using a Nikon Eclipse 80i epifluorescence microscope equipped with a 40x (1.30 NA) oil immersion objectives and a Texas Red HYQ filter cube for LTR imaging. Cells were grown on glass coverslips and maintained under normal growth conditions for 24 hours with or without 10 μ M imipramine. In the last 2 hours of treatment, 200 nM LTR was added to the cells. Cells undergoing ionophore treatment, had 10 μ M nigericin and 20 μ M monensin added just prior to the addition of

LTR. At the end of the 24-hour treatment, cells were washed three times with D-PBS rapidly and immediately mounted on glass microscopy slides for imaging. Images were acquired using an ORCA ER camera (Hamamatsu). Images were analyzed using Metamorph version 7.0 imaging software (Universal Imaging). Images were acquired under identical microscope settings and scaled identically to allow for comparison.

3.2.4. Amine accumulation assays

Cells were exposed to the indicated fluorescent or radiolabeled amine-containing compounds under normal growth conditions for 30 minutes to 2 hours depending on the study. LTR was added at a concentration of 200 nM unless otherwise noted, propranolol was added at a concentration of 100 nM containing 10 nM ^3H -propranolol and ^{14}C -methylamine was added at a concentration of 10 μM . Cells were then washed with D-PBS rapidly to prevent loss of cell-associated compound. Cells were lysed in either 0.1 M NaOH or lysis buffer (50 mM tris base, 150 mM NaCl, 1% NP40 adjusted to pH 7.4). Lysed samples were immediately assessed for fluorescence in RFU or radioactivity in CPM. Fluorescence was measured using a Bio-Tek FL600 microplate fluorescence reader equipped with 530 nm excitation and 590 nm emission filters. Radioactivity was measured using a Beckman LS 60001C liquid scintillation counter. Background signal contribution from non-specific binding of compound to polystyrene surfaces or from cell-associated signal was subtracted from the sample measurements. Cell protein content was measured using the BCA method, and RFU or CPM counts were normalized to cellular protein and overall cellular accumulation of each compound

was expressed as either RFU or CPM per microgram of protein or as a percentage of vehicle-treated control cells.

3.2.5. Drug uptake and release studies

Cells pretreated with 10 μ M imipramine or vehicle alone for 48 hours were exposed to 100 nM propranolol, containing 10 nM 3 H-propranolol and 90 nM unlabeled propranolol or 1 μ M LTG for various times up to 2 hours. At the designated timepoints, drug containing media was removed and cells were washed twice rapidly with D-PBS. Lysis buffer was added to each well and incubated at 37°C for 5 minutes. Cell lysate was transferred to ScintiVerse BD Cocktail (Fisher) for scintillation counting or a 96-well plate for fluorescence measurements and measured for protein content using the BCA method. In addition, a dose-titration experiment was conducted in imipramine treated cells to establish a dose of propranolol and LTG necessary to give equivalent cellular accumulation in vehicle treated (control) and imipramine treated cells (data not shown). Doses of 74 nM propranolol and 500 nM LTG were established in imipramine treated cells to obtain a similar level of uptake seen in vehicle treated cells dosed with 100 nM propranolol and 1 μ M LTG, respectively.

Following a 46-hour treatment with imipramine or vehicle alone, 100 nM propranolol (10% 3 H-propranolol) or 1 μ M LTG was added to vehicle-treated control cells and 74 nM propranolol (10% 3 H-propranolol) or 500 nM LTG was added to imipramine treated cells. Following a 2 hour incubation, the media was removed and cells were washed with 37°C D-PBS. Fresh media without propranolol or LTG was added to the cells. Imipramine or vehicle alone was maintained in the media through

the entirety of the experiment. For propranolol release, media was removed at each timepoint and transferred to scintillation fluid and fresh media was added. After the last timepoint, lysis buffer (pH 7.4, 50 mM tris base, 150 mM NaCl, 1% NP40) was added and incubated at 37°C for 5 minutes. Cell lysate was transferred to scintillation fluid and the remaining cell associated ^3H -propranolol was measured along with cell protein content using the BCA method. For LTG release, media was removed and lysis buffer was added at each timepoint. Release was measured over 2 hours and all remaining samples were washed at the 30-minute and 60-minute timepoints to maintain sink conditions. Cell lysate from each timepoint was transferred to a 96-well plate for fluorescence readings and protein determination by the BCA method. ^3H -propranolol in each sample was measured in DPM using a Beckman LS 60001C liquid scintillation counter. Fluorescence measurements in RFU were made using a Bio-Tek FL600 microplate fluorescence reader equipped with 485 nm excitation and 530 nm emission filters. Radioactivity and fluorescence counts were normalized to cellular protein content and plotted as cell-associated compound in DPM or RFU per microgram of protein as a function of time, respectively.

3.2.6. Lysosomal pH measurements

The influence of imipramine treatment on lysosomal pH was measured using a previously published protocol (Ndolo, Forrest et al. 2010) with slight modifications. Briefly, MDA-1986 cells were plated onto 8-chamber glass microscope slides and allowed to adhere overnight. Following a 24 hour exposure to 10 μM imipramine or vehicle alone, growth media was replaced with phenol red free growth media containing

1 mg/ml anionic 70,000 mol. wt. Oregon Green Dextran. Imipramine or vehicle alone was maintained in the media through the entirety of the experiment. Cells were incubated for 2 hours under normal growth conditions followed by removal of the fluorescent dextran conjugate containing media. Cells were washed twice with 37°C sterile D-PBS. Pre-warmed phenol red free growth media was added and cells were incubated under normal growth conditions for 6 hours to chase the dextran conjugated probe into lysosomes. Cells were then washed twice with and then maintained in a pH 7.4 buffer containing 150 mM NaCl, 20 mM Hepes, 5 mM KCl and 1 mM MgSO₄. Fluorescence intensity was measured while exciting at wavelengths of 451 nm and 495 nm on a microscope equipped with a Photon Technology International (Birmingham, NJ) Ratiomaster excitation spectrofluorometer, a D/F/TR multiple bandpass dichroic mirror, a 525/10 nm emission filter and a photomultiplier tube detector. Ratios of fluorescence intensity emissions at 525/10 nm (RFU_{495 nm}/ RFU_{451 nm}) were used to calculate lysosomal pH according to lysosomal pH calibration curves constructed in both imipramine treated and vehicle-treated control cells (data not shown). Calibration curves were created by measuring the fluorescence emission ratio of the dextran conjugated probe localized to lysosomes in cells exposed to pH 4.0, 5.0, 5.5 and 6.0 buffer containing 150 mM sodium chloride, 20 mM Mes, 5 mM potassium chloride and 1 mM magnesium sulfate supplemented with 10 µM nigericin and 20 µM monensin, as established previously (Altan, Chen et al. 1998). A linear fit of the calibration curves allowed calculation of the lysosomal pH from the resulting best-fit line equation.

3.2.7. Lysosomal volume calculations

The amount of LTR accumulated in cells by ion trapping-based accumulation in lysosomes was determined in vehicle-treated control cells and cells pretreated with 10 μ M imipramine for 24 hours using eq. 1:

$$D_{lys} = D_{total} - D_{nig / mon} \quad (1)$$

where D_{lys} represents the amount of LTR accumulated in cells by ion trapping, D_{total} represents the amount of LTR that accumulates under normal growth conditions and $D_{nig/mon}$ represents the amount of LTR that accumulates in cells following disruption of intracellular pH gradients with nigericin and monensin.

In eq. 2 the fold change in ion trapping-based accumulation of LTR following treatment with imipramine (ΔD_{lys}) is directly related to changes in both lysosomal volume (ΔV_{lys}) and lysosomal concentrations of LTR ($\Delta [D]_{lys}$).

$$\Delta D_{lys} = \Delta [D]_{lys} \times \Delta V_{lys} \quad (2)$$

Eq.3 was derived by de Duve (1974) in his original work detailing the ion trapping theory for weak base accumulation in lysosomes. The equation simply states that for a weakly basic compound, such as LTR, the concentration in lysosomes ($[D]_{lys}$) relative to the extracellular space ($[D]_{ext}$) is approximately equal to the ratio of the hydrogen ion concentration in the lysosome ($[H^+]_{lys}$) compared to the extracellular space ($[H^+]_{ext}$).

$$\frac{[D]_{lys}}{[D]_{ext}} \approx \frac{[H^+]_{lys}}{[H^+]_{ext}} \quad (3)$$

Rearrangement of eq. 3 yields eq. 4. Assuming that in the experiment the concentration of LTR in the extracellular space and the extracellular pH are held constant eq. 4 can be simplified to eq. 5. It can therefore be stated that changes in the

lysosomal concentration of LTR in lysosomes is directly proportional to the concentration of hydrogen ions in the lysosomes.

$$[D]_{lys} \approx \frac{[D]_{ext}}{[H^+]_{ext}} \times [H^+]_{lys} \quad (4)$$

$$\Delta[D]_{lys} \approx \Delta[H^+]_{lys} \quad (5)$$

Substituting eq. 5 into eq. 2 yields eq.6, which relates changes in LTR ion trapping in lysosomes to changes in lysosomal pH and/or lysosomal volume.

$$\Delta D_{lys} \approx \Delta[H^+]_{lys} \times \Delta V_{lys} \quad (6)$$

Direct measurements of lysosomal pH and ion trapping-based accumulation of LTR in vehicle-treated (control) and imipramine treated cells allowed for an approximation of the relative lysosomal volume change resulting from imipramine treatment.

3.3. Results and Discussion

The ability of CADs to induce a generalized lipidosis in vitro and in vivo has been extensively studied yet there remains little consensus regarding the therapeutic and/or toxicological ramifications of this drug-induced effect. This report was focused on how exposure to the lipidosis-inducing CADs increases the cellular accumulation of amine-containing substrates for ion trapping-based accumulation in lysosomes. It is further hypothesized that increased accumulation of these amine-containing drugs results from a CAD-induced expansion of the aqueous volume of the lysosomal compartment. If they are confirmed, these results would reveal a therapeutically important consequence

of CAD-induced lipidosis in the form of a distribution-based drug-drug interaction pathway.

3.3.1. Effect of pharmacologically unrelated CADs on the cellular accumulation of substrates for ion trapping in lysosomes

In Chapter 2 the model CAD, imipramine, caused an increase in the cellular accumulation of substrates for lysosomal ion trapping. The increase in lysosomotropic amine accumulation could conceivably be caused by a specific pharmacological effect of imipramine, such as modulation of cellular amine transporter activity. Alternatively, it could be generally attributed to the physicochemical attributes of the molecule (i.e., the fact that it is both cationic and amphiphilic). To discern these two possibilities evaluation of the propensity of other drugs to enhance the lysosomal sequestration of LTR following pretreatment were carried out. Specifically, a series of eight additional drugs similar to imipramine were evaluated; these additional drugs are both cationic and amphiphilic based on the physicochemical descriptors of logP and pKa (Ploemen, Kelder et al. 2004; Tomizawa, Sugano et al. 2006) but they have varying and known mechanism of actions. The molecular structure of each CAD and their predicted pKa and logP values are illustrated in Figure 3.1. Each compound is described as having a weakly basic amine with predicted pKa values ranging from 7.75 to 9.68. In addition, each compound is predicted to be relatively hydrophobic (logP > 2). Interestingly, all of the CADs regardless of their therapeutic application caused a significant increase in the cellular accumulation of LTR (Figure 3.2). This data suggests that CADs cause the hyperaccumulation of amine-containing substrates for lysosomal ion trapping as a class

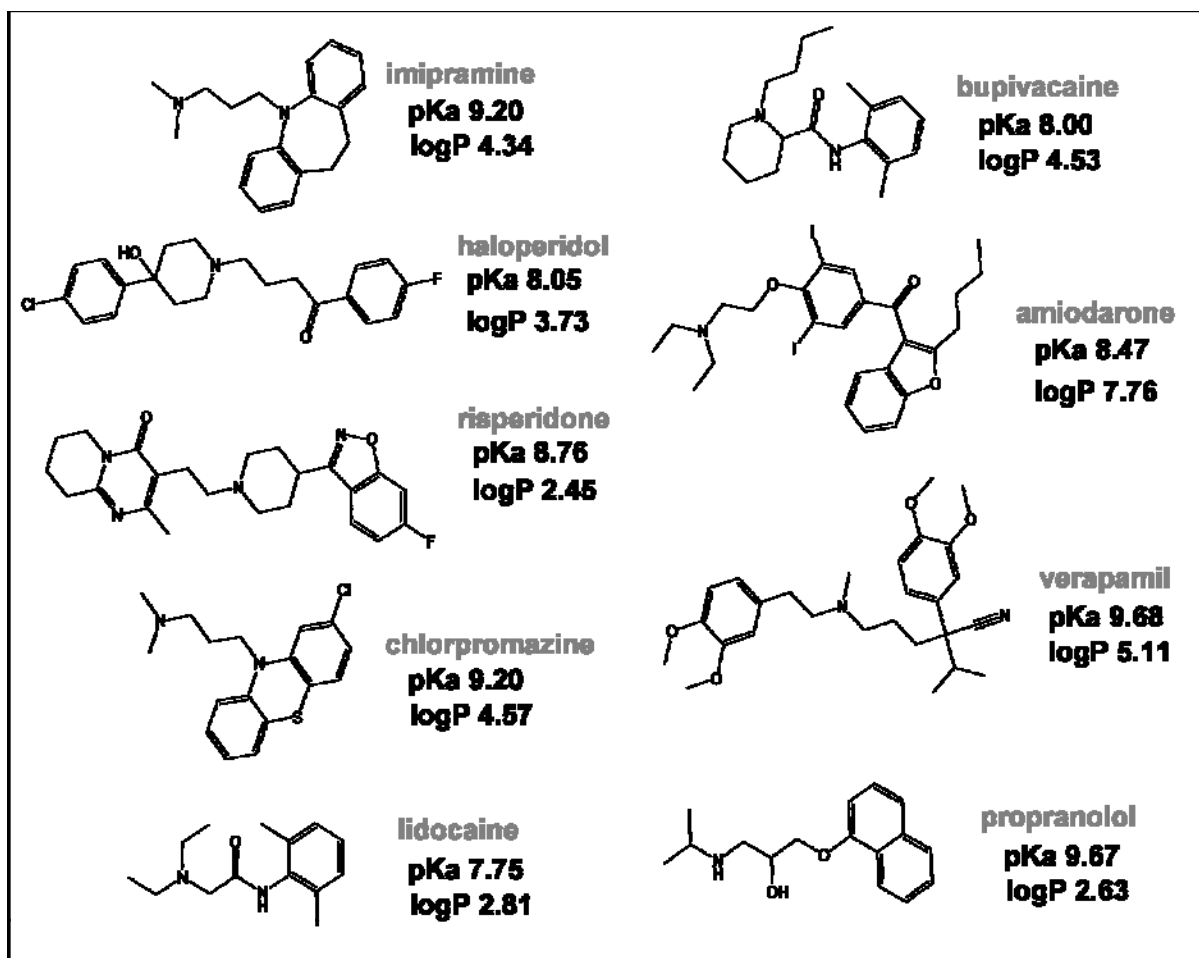


Figure 3.1. Structures and predicted physicochemical properties of model CADs. The CADs used in this study are listed along with their structure and predicted pKa and logP values. All CADs tested are relatively hydrophobic ($\log P > 2$) and contain a weakly basic amine with a pKa near neutrality ($\text{pKa} \geq 7$). The free software available from ChemAxon (<http://www.chemicalize.org>) was used to determine the predicted pKa and logP values for each compound.

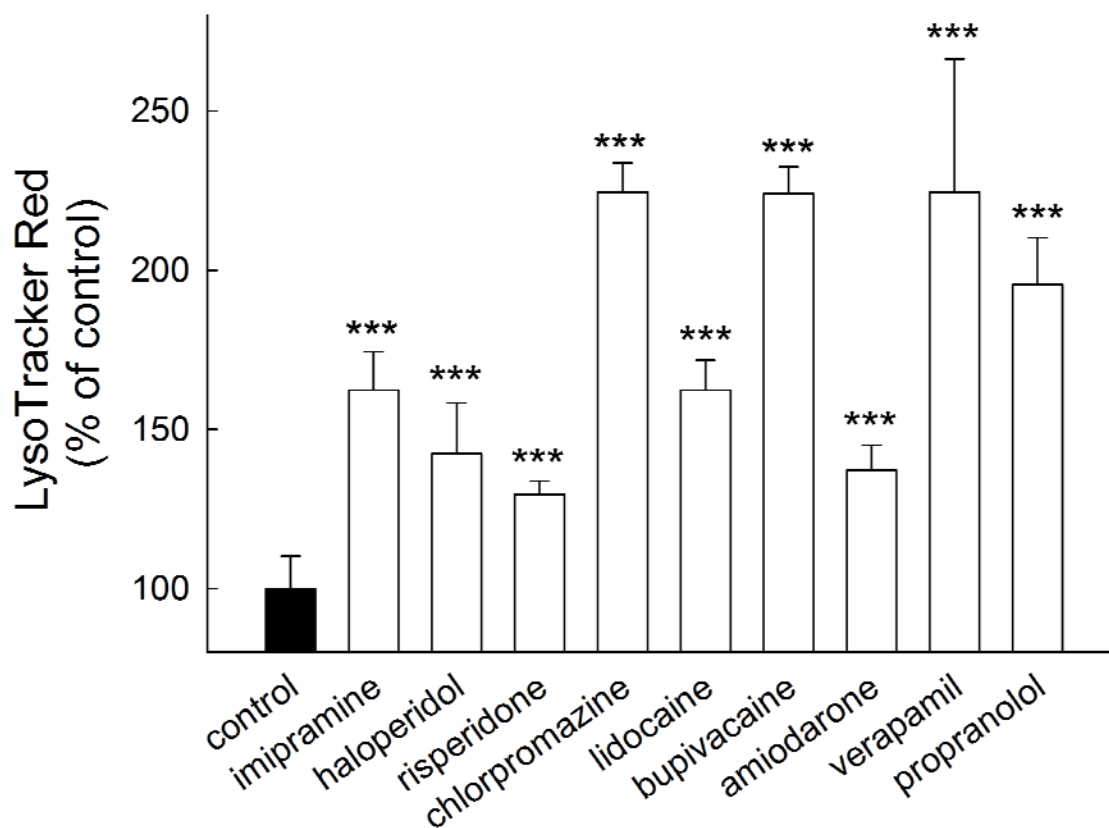


Figure 3.2. CADs increase the cellular accumulation of the model substrate for lysosomal ion trapping, LTR. MDA-1986 cells were treated with a variety of CADs from a diversity of pharmacological classes at a concentration of 10 μ M (white bars) or vehicle alone (black bar) for 24 hours. In the last 2 hours of treatment, 200 nM LTR was added to the treatment media. At the end of the treatment period cells were lysed and assessed for total cellular accumulation of LTR. All CAD treated cells accumulated significantly more LTR than control cells. Cellular accumulation of LTR was assessed by measuring fluorescence signal in the cell lysate and normalized to cellular protein content. Cellular accumulation of LTR was normalized to control cells and expressed as a percentage of control. Cellular accumulation of LTR is represented as mean \pm S.D. of at least three values for each sample (***, $p < 0.001$ by Student's t test).

effect related to the physicochemical properties of the molecule rather than its specific pharmacological activity. The significant difference in LTR accumulation observed with the different CADs may reflect the potency of these compounds in causing an expansion of the aqueous volume of the lysosome. Because the hypothesis is that lysosomal volume expansion results from the cellular lipidoses, the response may be reflective of the potency of the drug in inhibiting lysosomal lipid metabolism. Similarly, because CADs are amine-containing compounds they are also capable of causing mixed effects on the lysosome, such as alkalinization of lysosomes, and despite the expanded aqueous volume of the lysosome the concomitant alkalinization of the lysosomes would reduce the cellular accumulation of LTR.

The ability of CADs to increase the cellular accumulation of amine-containing drugs illustrates a pathway through which drug co-administration can lead to distribution-based pharmacokinetic drug-drug interactions. These results, at first glance, would appear to directly contradict earlier work that has illustrated the capacity of amine-containing drugs to inhibit the cellular uptake of each other by competing for uptake into lysosomes (Daniel and Wojcikowski 1999). This competition was presumed to occur through the ability of these drugs to buffer the lysosomal pH and inhibit the lysosomal uptake of secondarily applied amine-containing drugs. These discrepancies are believed to have originated from differences in drug dosing and duration of exposure leading to differential physiological effects on cells and the lysosomal system as illustrated by the dose-dependent effect of imipramine on the cellular accumulation of LTR, illustrated later in this chapter (Figure 3.18). These earlier studies relied on short-term, higher-dose drug treatments which are known to cause lysosomal alkalinization

(Ishizaki, Yokogawa et al. 2000) and therefore reduced ion trapping of amine-containing drugs in lysosomes without allowing the time-dependent expansion of the lysosomal compartment observed in this chapter, see Figure 3.14. This current study relied on longer durations of drug exposure with relatively lower treatment doses, which is more closely associated with the induction of a cellular lipidoses (Yoshida, Arimoto et al. 1985; Liscum and Faust 1989; Martin, Kachel et al. 1989). In fact, later in this chapter, experiments were carried out with treatments that are known to cause lysosomal alkalization (Ohkuma and Poole 1978; Woo, Shinohara et al. 1992) and it was found that they all significantly reduced the cellular accumulation of LTR in contrast to the ability of the CADs to increase LTR accumulation (Figure 3.17). Thus, the results here do not argue against previous observations of potential drug-drug interactions occurring through drug-induced lysosomal alkalization, but offer an additional pathway through which amine-containing drugs that accumulate in lysosomes can influence the pharmacokinetic properties of each other depending on their physiological effect on lysosomes. Since the therapeutic concentration of most CADs is well below the concentrations necessary to cause lysosomal alkalization and because most of the CADs are used as chronic therapies it could be argued that the CAD-induced lipidoses is a more likely clinical scenario than lysosomal alkalization. By enhancing the cellular retention of a secondarily applied drug, one would expect changes in the pharmacokinetic profile of that drug, such as an increase in its volume of distribution, through enhanced tissue accumulation, as well as, increased distribution to tissues enriched in lysosomes, such as the liver and lung. Without in vivo data, the potential for such a drug-drug interaction to be of therapeutic relevance is only speculative, but it is

possible that such changes in the macroscopic pharmacokinetic properties of a drug could result in altered therapeutic response.

3.3.2. Importance of intact intracellular pH gradients on the CAD-induced cellular accumulation of substrates for lysosomal ion trapping

In addition to the lipidosis caused by CADs, they are also known to cause changes in cellular membrane permeability (Tsao, Iga et al. 1982; Drori, Eytan et al. 1995), either of which could enhance cellular drug accumulation by providing additional intracellular binding sites for drugs or increasing permeation of drugs into cells, respectively. Specifically, CADs have been described as causing the cellular accumulation of phospholipid species, such as the anionic lysosomal phospholipid, lysobisphosphatidic acid (LBPA) (Yamamoto, Adachi et al. 1971). Like other phospholipids, LBPA has been proposed to bind lysosomotropic amine-containing drugs (Kolzer, Werth et al. 2004), most likely through charge-charge and hydrophobic interactions (Seydel and Wassermann 1976). Therefore, their accumulation within lysosomes may provide additional intralysosomal retention sites for these drugs. Previous studies in Chapter 2 indicated that non-lysosomotropic amines such as sulforhodamine 101 and MitoTracker Red did not experience enhanced accumulation in cells pretreated with CADs. These results were consistent with the notion that CAD-induced enhancement of amine uptake cannot simply be attributed to an increase in intracellular cationic drug binding sites or a change in cell membrane permeability, but rather suggested increased accumulation by ion trapping in lysosomes.

To confirm that the CAD-induced enhancement in amine accumulation was indeed reliant on intracellular pH gradients (i.e., lysosome to cytosol), the cellular accumulation of LTR in cells pretreated with the ionophores, nigericin and monensin was evaluated. When treated with ionophores, the pH of all cellular compartments equals that of the pH of the cell culture medium. Under these conditions no intracellular pH gradients exist and therefore no ion trapping-based accumulation can theoretically occur. Both vehicle-treated control cells and cells pretreated with the CAD, imipramine, were treated with ionophores and evaluated for differences in LTR distribution and accumulation. Interestingly, treatment of cells with ionophores negated any CAD induced enhancements in LTR accumulation. Fluorescence-based imaging showed an increase in the vesicular accumulation of LTR in cells treated with imipramine, which was abolished by ionophore treatment (Figure 3.3). Similarly, ionophore treatment eliminated the enhanced cellular accumulation of LTR seen in imipramine treated cells (Figure 3.4). These results further suggest that the CAD-induced enhancement in LTR accumulation is dependent upon ion trapping and cannot simply be attributed to an increase in binding sites or changes in membrane permeability in CAD treated cells.

Because intralysosomal lipid binding of amine-containing drugs is thought to result from both hydrophobic and ionic interactions, the ionized state of the lipid-species and the drug may be important in complex formation under physiological conditions. Since the ionization state is strongly influenced by pH, the formation of the lipid-drug complex may be extremely pH sensitive and might not have been observed under the experimental conditions in Figure 3.4. This was due to the ionophore treatment that was conducted with an extracellular pH of 7.4 that is far greater than the normal

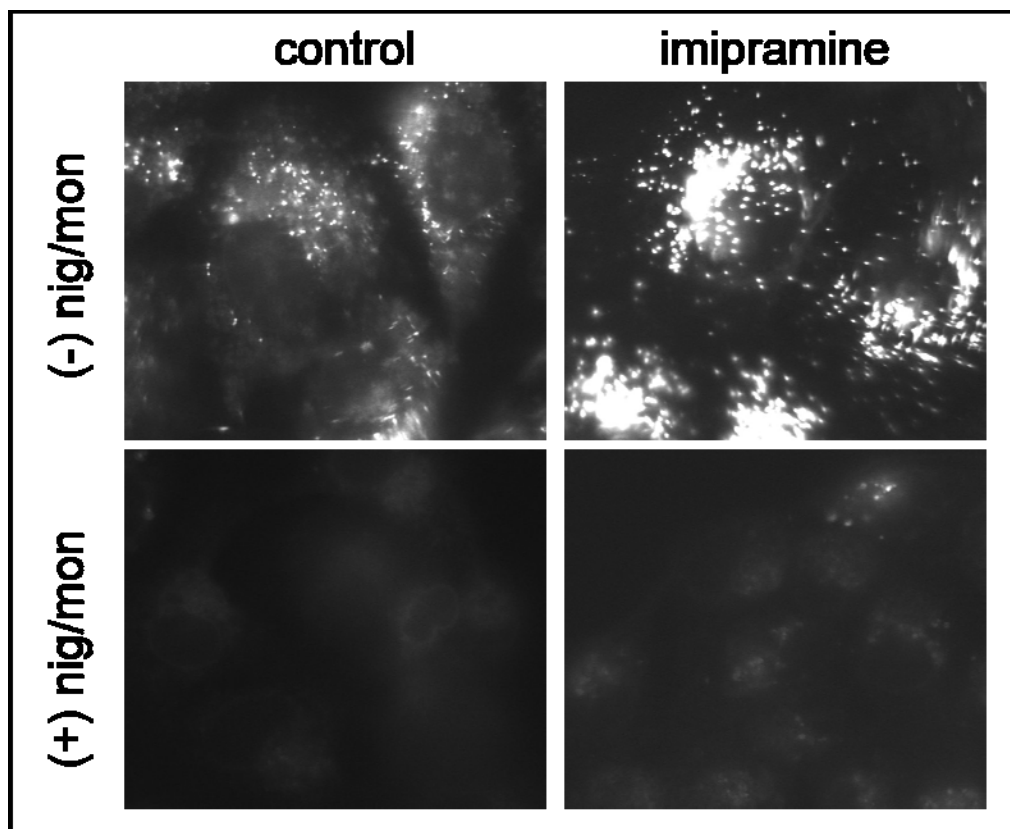


Figure 3.3. Disruption of intracellular pH gradients reverses the CAD-induced increase in vesicular accumulation of LTR. MDA-1986 cells were treated with 10 μ M imipramine or vehicle alone (control) for 24 hours. In the last 2 hours of treatment, ionophores (10 μ M nigericin and 20 μ M monensin) were added to eliminate intracellular pH gradients ((+) nig/mon) or the cells were left untreated ((-) nig/mon). In addition, 200 nM LTR was added immediately following the addition of ionophores. After 2 hours under normal growth conditions, cells were washed and imaged. The increase in vesicular accumulation of LTR in imipramine treated cells was reversed by ionophore treatment indicating that intact intracellular pH gradients are important in the imipramine-induced increase in LTR accumulation.

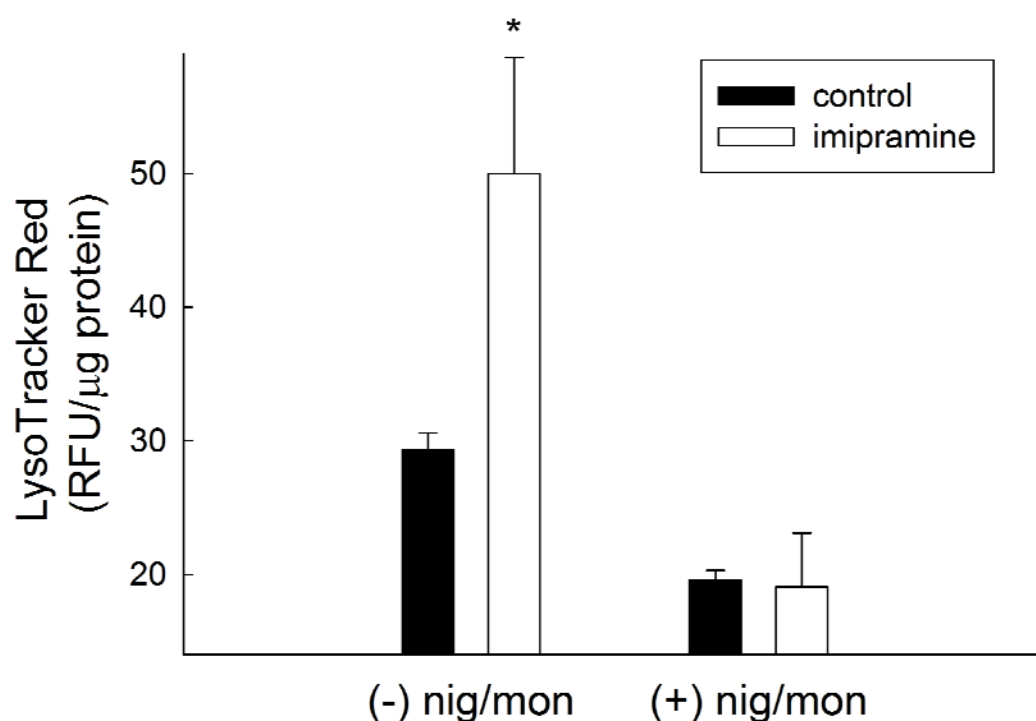


Figure 3.4. Disruption of the intracellular pH gradient reverses the CAD-induced cellular accumulation of LTR. MDA-1986 cells were treated with 10 μ M imipramine (white bars) or vehicle alone (black bars) for 24 hours. In the last 2 hours of treatment, ionophores (10 μ M nigericin and 20 μ M monensin) were added to eliminate intracellular pH gradients ((+) nig/mon) or the cells were left untreated ((-) nig/mon). In addition, 200 nM LTR was added immediately following the addition of ionophores. After 2 hours under normal growth conditions, cells were lysed and LTR accumulation was assessed by measuring cell associated fluorescence signal. Without ionophores, imipramine treatment resulted in a significant increase in the amount of LTR accumulated in cells. Ionophore treatment decreased LTR accumulation to similar levels in control and imipramine treated cells. Cellular accumulation of LTR is represented in fluorescence counts (RFU) per μ g of protein as mean \pm S.D of three values for each sample (*, $p < 0.05$ by Student's t test).

lysosomal pH of 5.0. To examine the possibility of enhanced pH-dependent lipid binding following imipramine treatment, the pH-dependent cellular binding of the lysosomotropic amines LTR and propranolol was measured. LTR and propranolol accumulation was measured in imipramine and vehicle-treated control cells following ionophore treatment in pH controlled solutions. Ionophore treatment dissipates cellular pH gradients and equilibrates the intracellular pH with the extracellular media. Therefore if imipramine causes an increase in pH-sensitive intralysosomal lipid binding of LTR or propranolol it should be observed at the lower pH. Imipramine treatment failed to affect LTR accumulation at pH 7.3, 6.0 or 5.0 (Figure 3.5). Similarly, imipramine treatment failed to increase propranolol accumulation at pH 5.0 (Figure 3.6), which is the normal lysosomal pH of this cell line. Together these results suggest that increased intralysosomal binding is in fact not the mechanism for enhanced retention of these drugs following imipramine treatment.

Additionally, the hydrophilic substrate for lysosomal ion trapping, methylamine would not be expected to participate in the hydrophobic interactions predicted for other more hydrophobic amine-containing compounds, such as propranolol, and would perhaps serve exclusively as a measure of cellular uptake through ion trapping. Therefore, the ability of imipramine to affect the cellular accumulation of methylamine was assessed (Figure 3.7) and found to be significantly increased, further suggesting that increased accumulation of the amine-containing compounds occurs through an increase in lysosomal ion trapping capacity of cells mediated by CAD exposure.

Together, these results argue that although the lipidosis may be important in the CAD-induced enhancement in accumulation of substrates for lysosomal ion trapping,

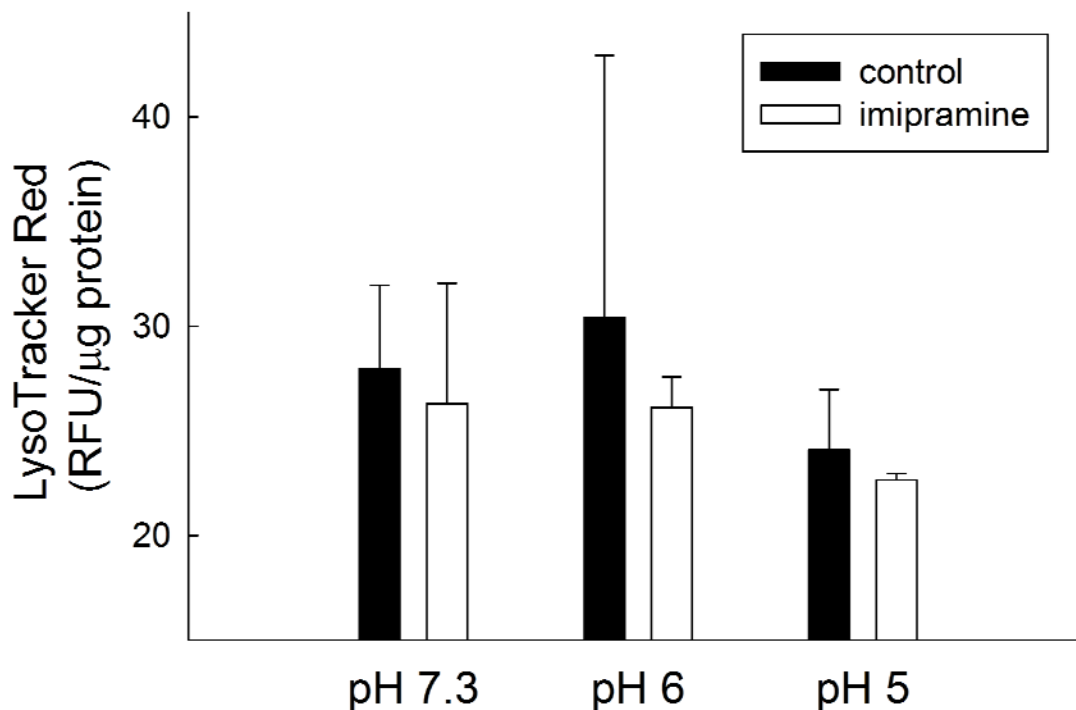


Figure 3.5. CAD treatment does not increase the cellular binding of LTR in a pH-sensitive manner. MDA-1986 cells were treated with 10 μ M imipramine (white bars) or vehicle alone (black bars) for 24 hours. Cells were subsequently placed in isotonic solutions buffered at pH 5.0, 6.0 and 7.3 along with ionophores (10 μ M nigericin and 20 μ M monensin) to allow equilibration between intracellular pH and extracellular pH. Following the addition of ionophore, 200 nM LTR was added. After 30 minutes cells were lysed and LTR accumulation was assessed by measuring cell associated fluorescence signal. Without an intact intracellular pH gradient lysosomal ion trapping doesn't occur, therefore cellular accumulation is expected to represent cellular binding of LTR. LTR didn't show a pH-sensitive difference in cellular binding in control versus imipramine treated cells. Cellular accumulation of LTR is represented in fluorescence counts (RFU) per μ g of protein as mean \pm S.D of three values for each sample.

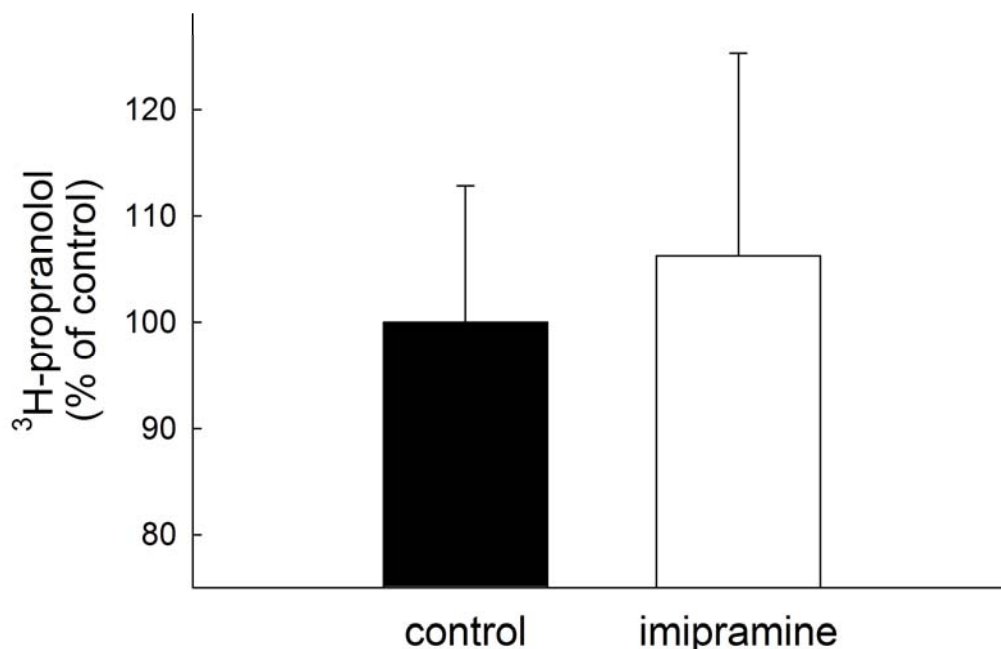


Figure 3.6. CAD treatment does not increase the cellular binding of propranolol in a pH-sensitive manner. MDA-1986 cells were treated with 10 μ M imipramine (white bar) or vehicle alone (black bar) for 24 hours. Cells were subsequently placed in an isotonic solution buffered at pH 5.0 along with ionophores (10 μ M nigericin and 20 μ M monensin) to allow equilibration between intracellular pH and extracellular pH. Following the addition of ionophore, 100 nM propranolol containing 10 nM ³H-propranolol was added. After 2 hours cells were lysed and ³H-propranolol accumulation was assessed by measuring cell associated radioactivity and normalized to cellular protein content. Without an intact intracellular pH gradient lysosomal ion trapping doesn't occur; therefore, cellular accumulation is expected to represent cellular binding of propranolol. Propranolol didn't show an increase in cellular binding at pH 5.0 in control versus imipramine treated cells. Cellular accumulation of propranolol as a percentage of the vehicle-treated control was determined and is plotted as the mean \pm S.D of three values for each sample.

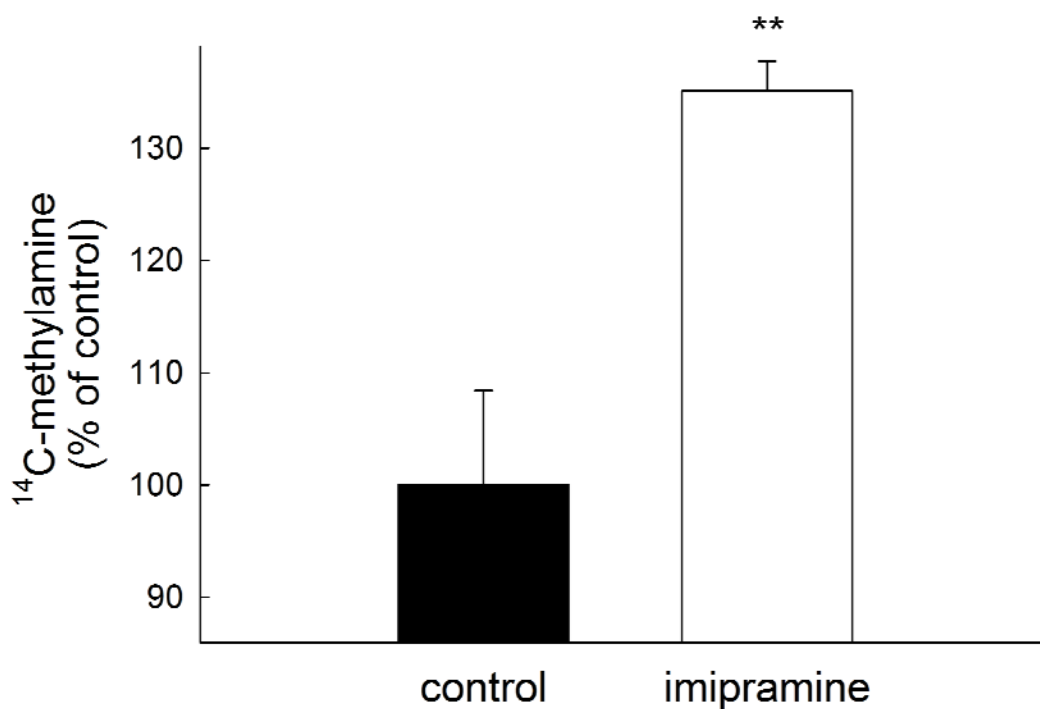


Figure 3.7. CAD treatment increases the cellular accumulation of the hydrophilic amine, methylamine. MDA-1986 cells were treated with 10 μM imipramine (white bars) or vehicle alone (black bars) for 24 hours. In the last 2 hours of treatment, 10 μM ^{14}C -methylamine was added. After 2 hours cells were lysed and ^{14}C -methylamine accumulation was assessed by measuring cell associated radioactivity and normalized to cellular protein content. Imipramine treatment caused a significant increase in the cellular accumulation of the hydrophilic amine, methylamine. Cellular accumulation of methylamine as a percentage of control is plotted as the mean \pm S.D of three values for each sample (**, $p < .01$ by Student's t test).

that an increase in cellular binding of the drug to lipids, or other cellular constituents, is not the mechanism of increased cellular accumulation. The dependence on intact intracellular pH gradients further suggests that imipramine increases the capacity of cells to accumulate amine-containing drugs by ion trapping in lysosomes, either through changes in lysosomal pH or lysosomal volume. These results are in agreement with previous *in vivo* and *in vitro* findings that the CAD-induced lipidosis causes an increase in the tissue and cellular accumulation of a secondarily administered amine-containing drug (Lullmann, Rossen et al. 1973; Hein, Lullmann-Rauch et al. 1990; Honegger, Zuehlke et al. 1993), but in addition to the previous observation it was demonstrated that increased drug accumulation does not occur through increased lipid binding. Here it is argued that, in contrast to increased cellular drug binding, CADs cause an expansion of the lysosomal compartment, and therefore increase the cellular accumulation of amine-containing drugs through an increase in the lysosomal ion trapping capacity of cells.

3.3.3. CAD-induced lipidosis and enhanced lysosomal ion trapping

A similarity in the pharmacological response of imipramine in causing both a cellular lipidosis and enhanced accumulation of substrates for ion trapping in lysosomes suggests a possible relationship between the two events. Although the two effects could be independent of one another, it is likely that the hypothesized CAD-induced expansion of the lysosomal compartment is secondary to the lysosomal accumulation of lipids. If the expanded lysosomal compartment is indeed secondary to the drug-induced lipidosis, then reversal of the lipidosis would also be expected to reverse the lysosomal

volume expansion. To test this hypothesis, several treatments that prevent the lipidosis were employed, including 0.1% HPCD (Abi-Mosleh, Infante et al. 2009) and growth in lipoprotein depleted media (i.e., LPDS) (Thomas, Tuck-Muller et al. 1989). Interestingly, both treatments showed a significant reduction in the cellular accumulation of LTR following imipramine treatment resulting in levels similar to that seen in vehicle-treated control cells (Figure 3.8). In addition, growth in LPDS also reduced the cellular accumulation of LTR in NPC disease cells, NPC1^{-/-} and NPC2^{-/-} (Figure 3.9).

Together these results support the hypothesis that the lipidosis, whether mediated by CAD treatment or through inborn errors in lipid metabolism, results in the enhanced accumulation of substrates for lysosomal ion trapping, presumably through an expansion of the lysosomal compartment. The expansion of the lysosomal compartment may result simply from the accumulation of undigested membranous lipid material in lysosomes or may reflect a generalized defect in the vesicle-mediated trafficking of lysosome, as has been previously hypothesized in CAD treated (Kaufmann and Krise 2008) and NPC disease cells (Neufeld, Wastney et al. 1999). If a blockage of the outflow of lysosomes and their cargoes were to occur, an increase in the resting volume of the lysosomal compartment may be expected. The ability of lipid depletion to at least partially reverse the apparent lysosomal volume expansion in both CAD treated and NPC disease cells would suggest that the trafficking defect is secondary to the accumulation of lipids and may not be a direct result of inhibition of vesicle-mediated trafficking by CADs or dysfunctional NPC1 or NPC2. Previous findings that vesicle-mediated efflux trafficking of the lysosomal cargo, ¹⁴C-sucrose, is inhibited by the lysosomal accumulation of cholesterol (Neufeld, Wastney et al. 1999) would support the

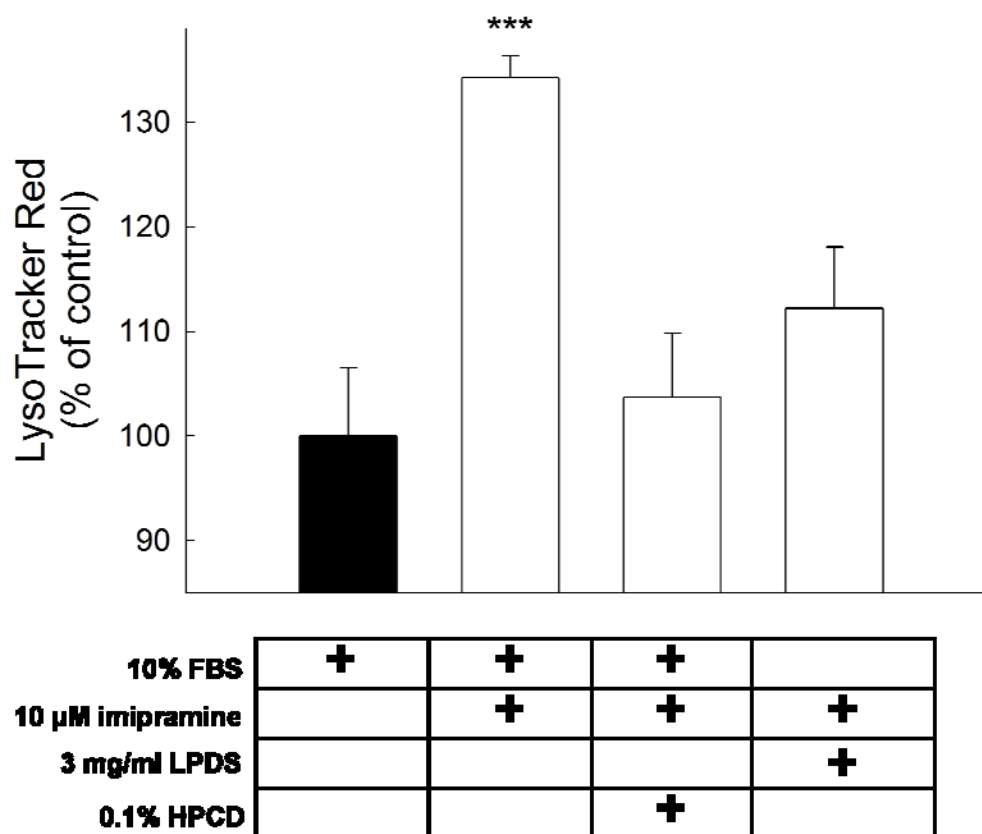


Figure 3.8. Inhibitors of the cellular lipidoses prevent the CAD-induced enhancement in lysosomal ion trapping capacity. MDA-1986 cells were treated with either normal cell growth media, normal growth media supplemented with 0.1% HPCD or growth media without FBS, but supplemented with 3 mg/ml LPDS, as indicated in the figure. Following 24 hours of growth under the indicated conditions, 10 µM imipramine (white bars) or vehicle alone (black bars) was added for a 24-hour treatment. In the last 2 hours of treatment, 200 nM LTR was added to the media. At the end of the experiment, cells were lysed and cell associated fluorescence was measured and normalized to cellular protein content. Despite the ability of imipramine to increase the cellular accumulation of LTR under normal cell growth conditions, pretreatment with 0.1% HPCD or growth in LPDS-containing media prevented this increase. Cellular accumulation of LTR as a percent of the vehicle-treated control cells is represented as mean \pm S.D of three values for each sample (***, $p < .001$ by Student's t test).

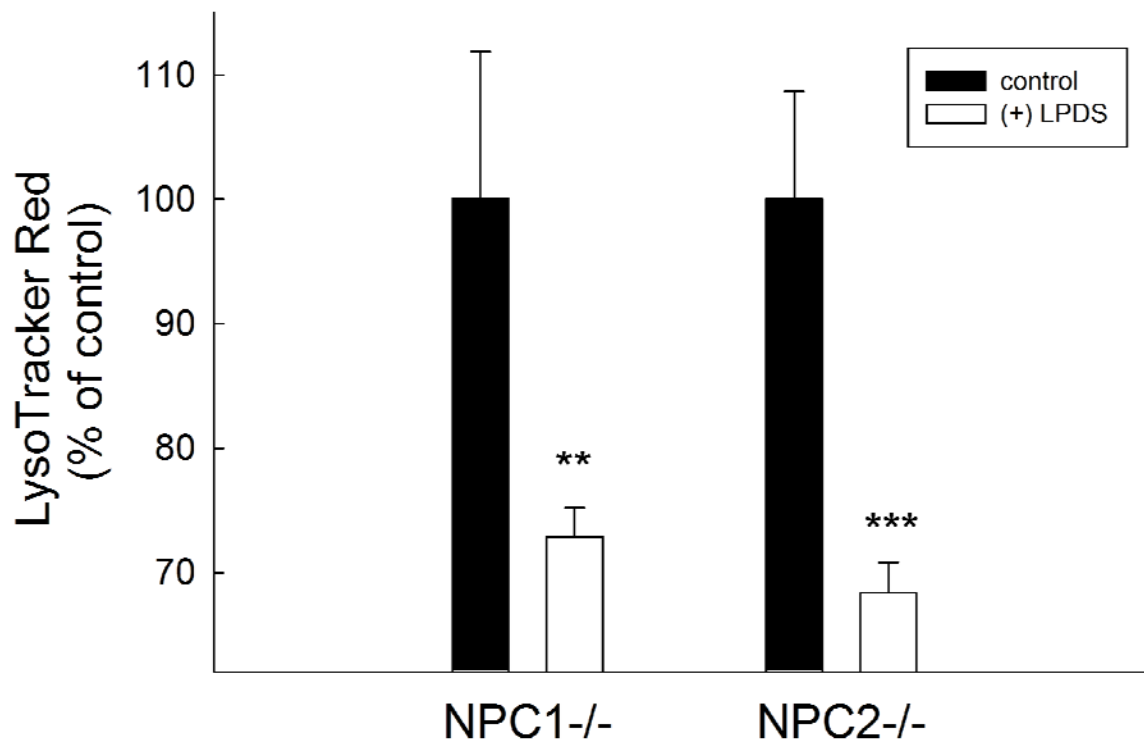


Figure 3.9. Reversal of the cellular lipidosis in NPC disease cells reduces the lysosomal ion trapping capacity. NPC disease fibroblasts with either loss-of-function mutations in NPC1 (NPC1^{-/-}) or NPC2 (NPC2^{-/-}) were treated for 72 hours with either normal cell growth media (black bars) or growth media without FBS, but supplemented with 3 mg/ml LPDS (white bars). In the last 2 hours, 200 nM LTR was added to the media. At the end of the experiment, cells were lysed and cell associated fluorescence was measured and normalized to cellular protein content. Growth in LPDS-containing media decreased LTR accumulation in both NPC disease cell lines. Cellular accumulation of LTR as a percent of the control cells in normal growth media for each cell line was determined and is represented as mean \pm S.D of three values for each sample (**, $p < .01$; ***, $p < .001$ by Student's *t* test).

hypothesis that lysosomal lipid accumulation may in fact result in an expanded lysosomal compartment through a blockage in the vesicle-mediated trafficking of lysosomes.

3.3.4. Influence of CAD treatment on the cellular uptake and release of substrates for lysosomal ion trapping

Having established that CADs cause an increase in cellular accumulation of substrates for ion trapping-based accumulation, the potential basis for this was examined further. Although numerous mechanisms of increased accumulation may exist, an understanding of the impact of CAD treatment on the uptake and release of the amine-containing compounds will facilitate a more thorough understanding of the basis for this apparent intracellular interaction. Lysosomotropic amines have a complicated release mechanism, including the potential for vesicle-mediated efflux (Michalik, Pierzchalska et al. 2003; Gong, Duvvuri et al. 2006), transporter mediated efflux (Willingham, Cornwell et al. 1986) and simple passive-diffusion (Dudley and Brown 1995). Since enhanced intralysosomal drug binding was not observed, it is reasoned that increases in drug uptake following CAD exposure would be indicative of an increase in the capacity of the cell to ion trap the drug in lysosomes, either through a change in the pH or volume of the lysosomal system. Decreases in drug release following CAD exposure would be indicative of either inhibition of transporter-mediated or vesicle-mediated drug efflux. Using the model substrates for lysosomal ion trapping, propranolol and LTG, it was found that imipramine treatment causes a profound increase in the uptake of both propranolol (Figure 3.10) and LTG (Figure 3.12), but

shows no inhibition of release for neither propranolol (Figure 3.11) nor LTG (Figure 3.13). These results indicate that CADs increase the uptake of drugs that accumulate by ion trapping without significantly influencing release. Increased uptake is presumed to reflect an increase in the lysosomal ion trapping of the drugs and is dependent on both the volume and pH of the lysosomal compartment.

3.3.5. Time- and temperature-dependence of enhanced cellular accumulation of substrates for lysosomal ion trapping following CAD treatment

Based on the theory of ion trapping, the extent of lysosomal accumulation depends on the physicochemical properties of the drug (i.e., pKa and α), as well as, the properties of the lysosomal system, such as pH and volume (de Duve, de Barsey et al. 1974). Since the inherent physicochemical properties of the drug are not influenced by CAD treatment it is assumed that the properties of the lysosomes must be altered. Therefore, the effect of imipramine treatment on lysosomal pH was measured and it was found that exposure to 10 μ M imipramine for 24 hours resulted in no significant change in lysosomal pH (pH 5.0 ± 0.9), as compared to vehicle-treated control cells (pH 4.9 ± 0.2).

The lack of a change in lysosomal pH following CAD treatment further supports the hypothesis that CADs are causing an expansion of the lysosomal compartment. This compartment volume expansion is suspected to result from a cellular remodeling process, such as through the inhibition of the vesicle-mediated efflux trafficking of

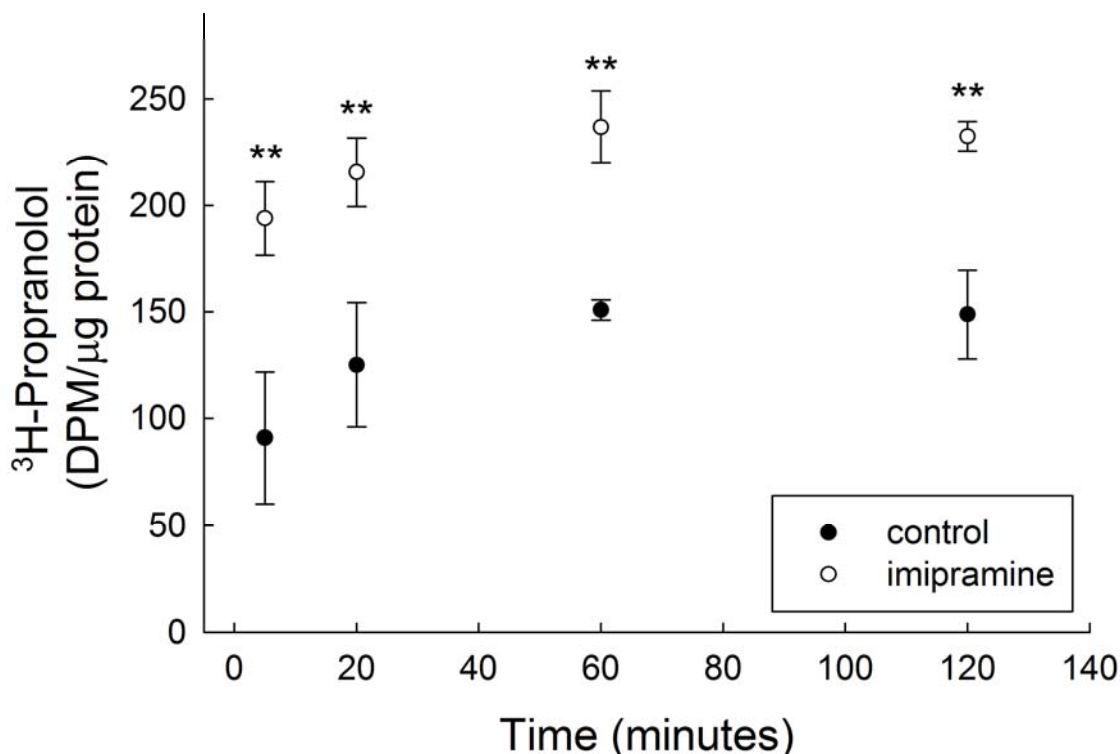


Figure 3.10. Enhanced uptake of substrates for lysosomal ion trapping following CAD treatment. Fibroblasts were treated with 10 μ M imipramine (white circles) or vehicle alone (black circles) for 48 h. In the last 2 hours 100 nM propranolol containing 10 nM 3 H-propranolol was added to the cell media for exposure times of up to 120 minutes. Cells were lysed and measured for associated 3 H-propranolol and normalized to cellular protein content. Cellular uptake of propranolol was rapid with the majority occurring in the first 5 minutes and steady state levels were achieved within 1 hour. Despite the similarity in the uptake profile of propranolol in both imipramine treated and control cells, imipramine treated cells took up significantly more propranolol at each timepoint. Cellular accumulation of 3 H-propranolol was measured in radioactivity counts (DPM) and normalized to cellular protein in μ g and is represented as mean \pm S.D of three values for each sample (**, $p < 0.01$ by Student's t test).

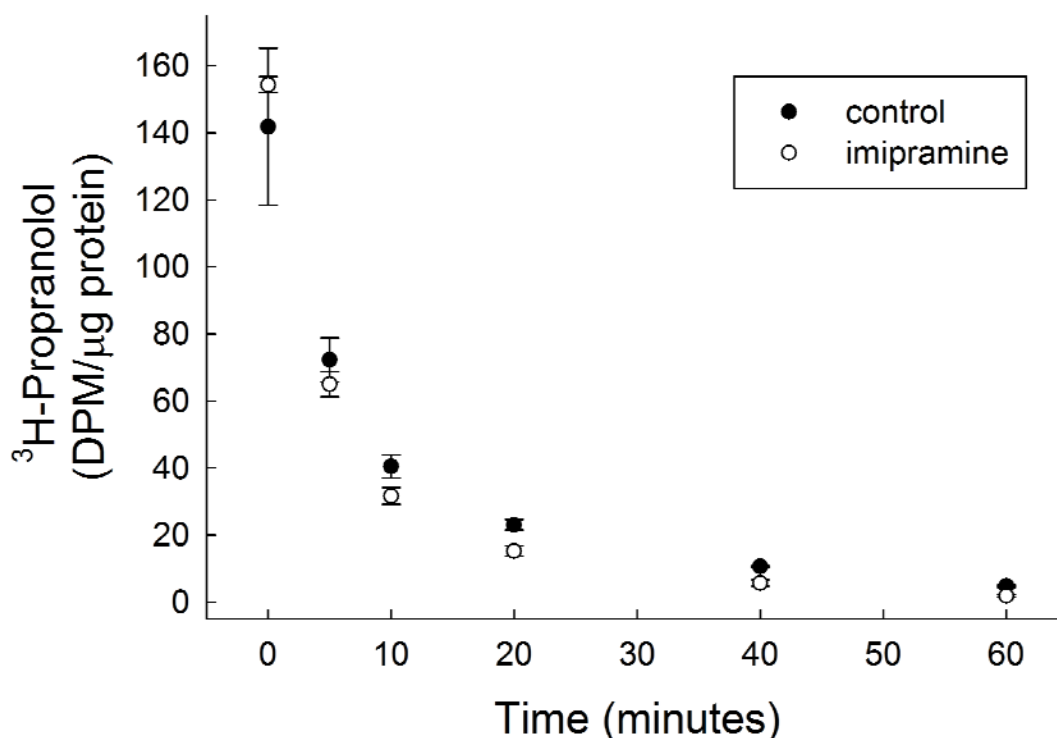


Figure 3.11. CAD treatment does not inhibit the release of substrates for lysosomal ion trapping. Fibroblasts were treated with 10 μM imipramine (white circles) or vehicle alone (black circles) for 48 hours. In the last 2 hours, 100 nM propranolol containing 10 nM ³H-propranolol and 74 nM propranolol containing 7.4 nM ³H-propranolol was added to vehicle-treated and imipramine treated cells, respectively. Following the 2 hour treatment period the propranolol release into the media was measured. Imipramine or vehicle alone was maintained in the release media. The cell media was removed at timepoints up to 1 hour and replaced with fresh media to maintain sink conditions. After the last timepoint, cells were lysed. Cell lysate and media samples were measured for ³H-propranolol content to determine total cellular accumulation and the release profile. ³H-propranolol was normalized to cellular protein content in the cell lysate. Imipramine treated cells showed a rapid release of propranolol that was similar to vehicle-treated control cells. Cell associated ³H-propranolol was measured in radioactivity counts (DPM) and normalized to cellular protein in μg and is represented as mean ± S.D of three values for each sample.

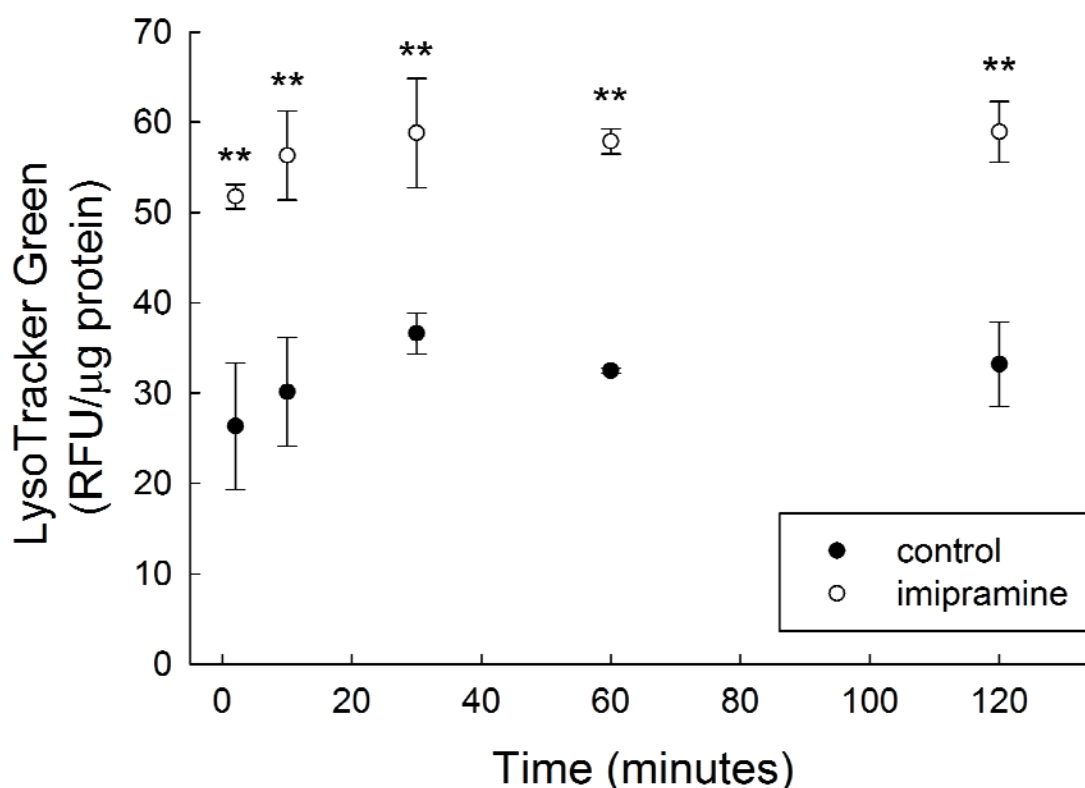


Figure 3.12. Enhanced uptake of substrates for lysosomal ion trapping following CAD treatment. Fibroblasts were treated with 10 μ M imipramine (white circles) or vehicle alone (black circles) for 48 hours. In the last 2 hours 1 μ M LTG was added to the cell media for exposure times of up to 120 minutes. Cells were lysed and measured for associated LTG and normalized to cellular protein content. Cellular uptake of LTG was rapid with the majority occurring in the first 5 minutes and steady state levels were achieved within 30 minutes. Despite the similarity in the uptake profile of LTG in both imipramine treated and control cells, imipramine treated cells took up significantly more LTG at each timepoint. Cellular accumulation of LTG was measured by fluorescence (RFU) and normalized to cellular protein in μ g and is represented as mean \pm S.D of three values for each sample (**, $p < 0.01$ by Student's t test).

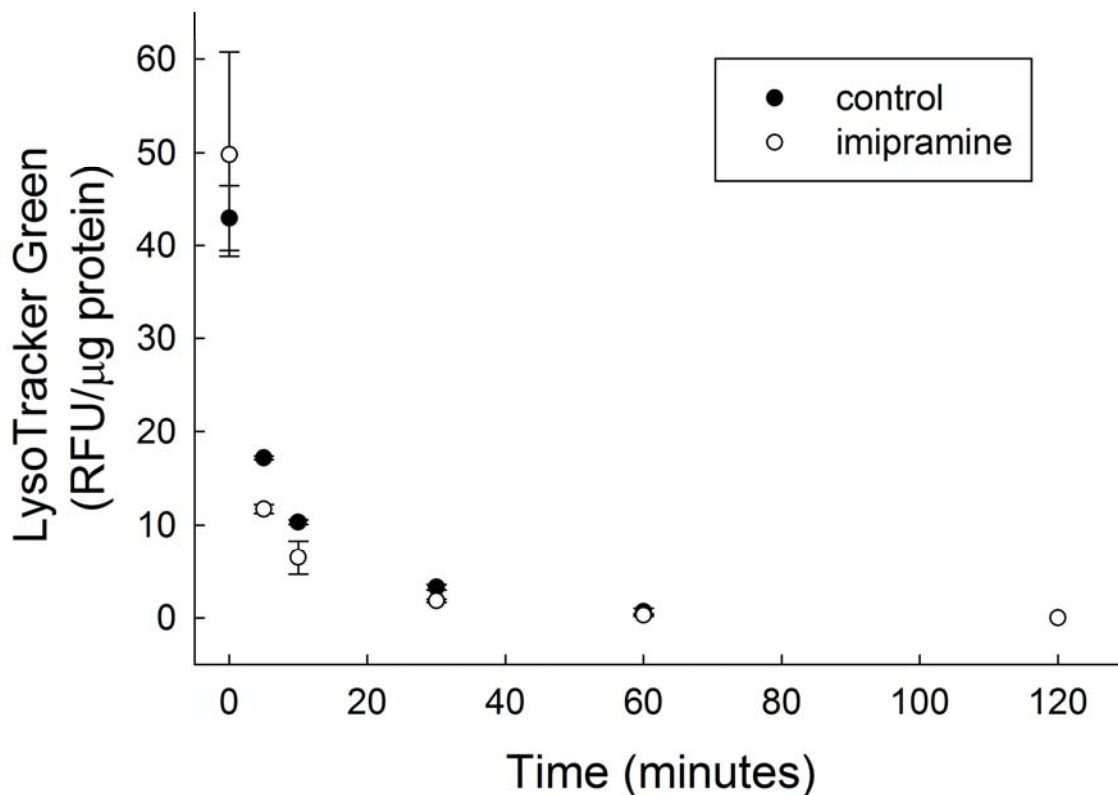


Figure 3.13. CAD treatment does not inhibit the release of substrates for lysosomal ion trapping. Fibroblasts were treated with 10 μ M imipramine (white circles) or vehicle alone (black circles) for 48 hours. In the last 2 hours, 1 μ M and 0.5 μ M LTG was added to vehicle treated and imipramine treated cells, respectively. Following the 2-hour treatment period the LTG release was measured. Imipramine or vehicle alone was maintained in the release media. The cell media was removed at timepoints up to 2 hours and cells were subsequently lysed. Media was replaced at 30 minutes and 60 minutes to maintain sink conditions. Cell lysate from each timepoint was measured for LTG content. Cellular accumulation of LTG, as indicated by the initial timepoint, was not significantly different in vehicle-treated and imipramine treated cells. Imipramine treated cells showed a rapid release of LTG that was similar to vehicle-treated control cells. Cell associated LTG was measured in fluorescence counts (RFU) and normalized to cellular protein in μ g and is represented as mean \pm S.D of three values for each sample.

lysosomes. Such a process is not expected to occur instantaneously, but would be expected to be dependent on both time and temperature. To test this possibility, cells were incubated with 10 μ M imipramine for varying amounts of time and the impact on LTR accumulation was measured. It was found that the CAD-induced enhancement in LTR was a progressive process with enhanced uptake minimal at early incubation times and maximal after approximately 10 to 15 hours of exposure (Figure 3.14).

Moreover, it was found that incubation of cells with 10 μ M imipramine for 4 hours failed to enhance the accumulation of LTR unless the temperature of the incubation was maintained at 37°C. Cells incubated with or without CAD at 4°C for 4 hours showed no differences in LTR accumulation at 37°C as compared to cells maintained at 37°C throughout the treatment period (Figure 3.15). These results are important since they are consistent with the notion that CADs impart some type of time- and temperature-dependent restructuring of the cells that facilitates the enhancement of LTR ion trapping-based accumulation and draws a correlation between the CAD-induced lipidosis and the observed increase in apparent lysosomal volume. These results along with the propranolol and LTG release studies would argue against the possibility that imipramine is in some way inhibiting an acute cellular efflux pathway for LTR and other weakly basic drugs, but rather is consistent with imipramine inducing an energy-dependent cellular remodeling process that results in an increased lysosomal volume and therefore enhanced accumulation of weakly basic drugs.

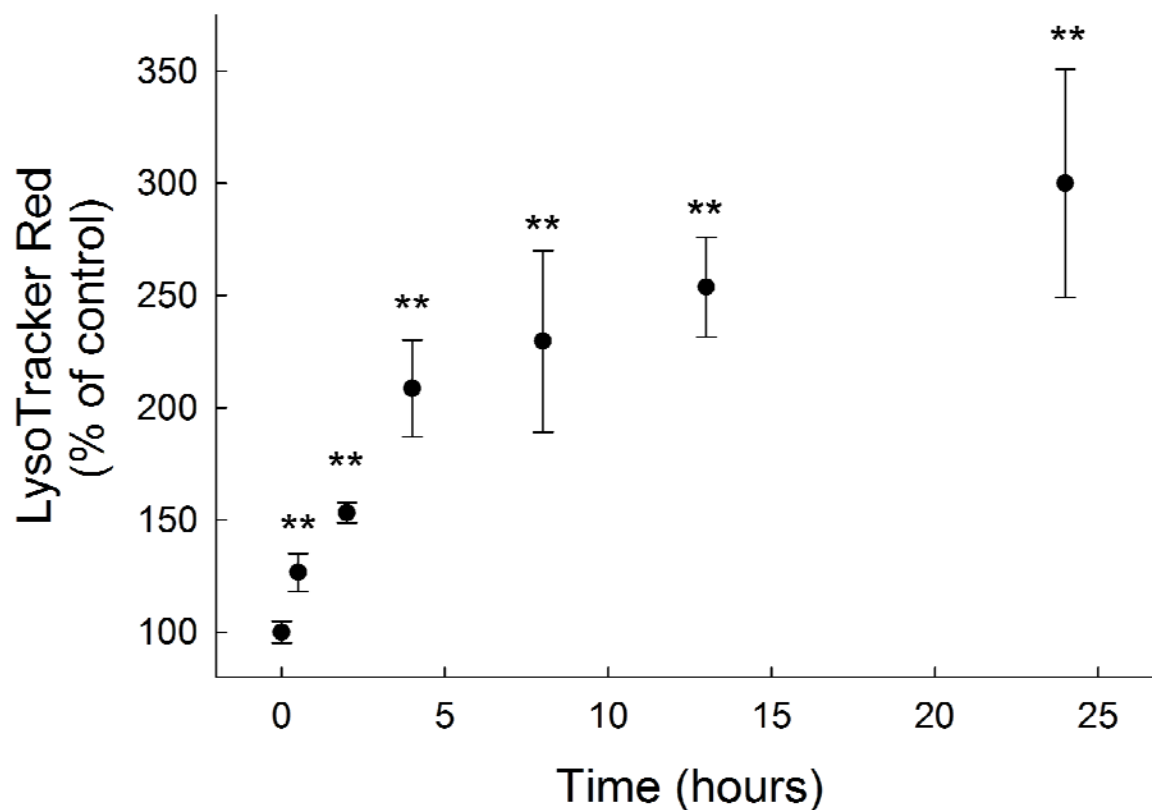


Figure 3.14. CAD-induced enhancement in cellular ion trapping capacity is time-dependent. MDA-1986 cells under normal growth conditions were treated with 10 μ M imipramine for durations of 0 to 24 hours. LTR at a concentration of 200 nM was added for the last 2 hours of treatment and cells were subsequently lysed. Cellular accumulation of LTR was determined by fluorescence measurements of the cell lysate. Fluorescence signal was normalized to cell lysate protein content. Cellular accumulation of the model substrate for ion trapping-based accumulation, LTR, is dependent on the duration of exposure to imipramine. A progressive increase in the ion trapping capacity of MDA-1986 cells was observed with imipramine treatment times of up to 24 hours. Cellular accumulation of LTR as a percent of the vehicle-treated control cells is represented as mean \pm S.D of three values for each sample (**, $p < 0.01$ by Student's t test).

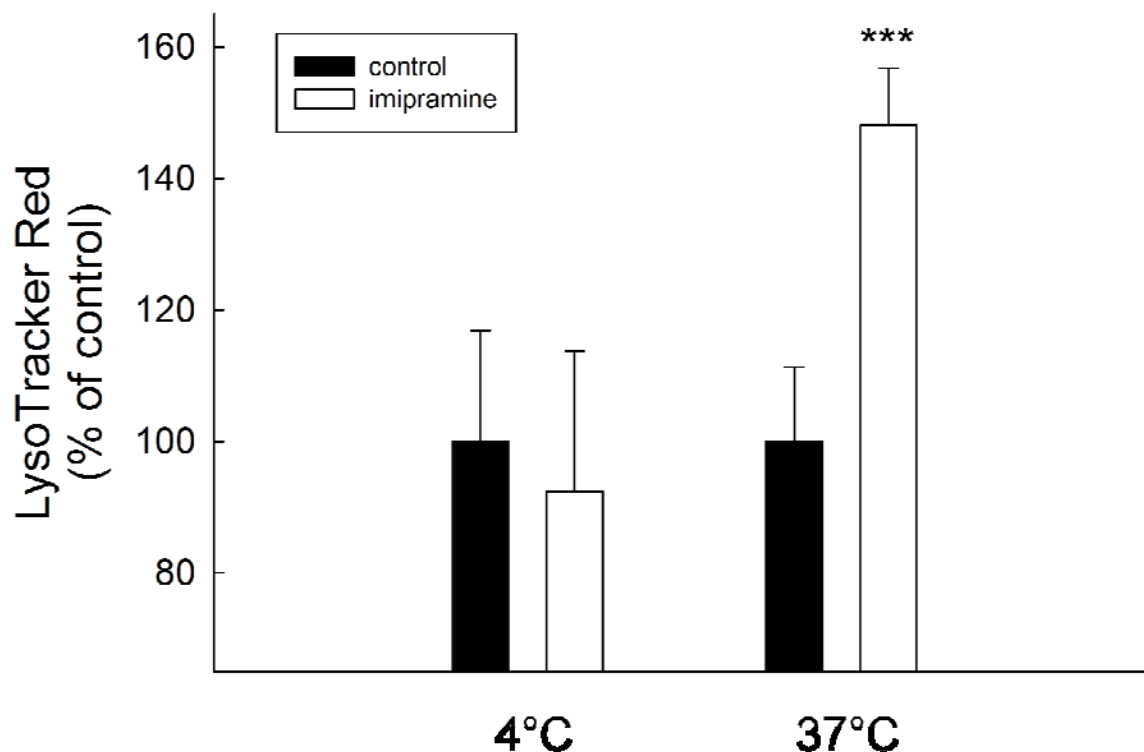


Figure 3.15. CAD-induced enhancement in cellular ion trapping capacity is temperature-dependent. MDA-1986 cells grown under normal culture conditions were subsequently exposed to a 4 hour treatment with 10 μ M imipramine (white bars) or vehicle alone (black bars) at either 37°C or 4°C, as indicated in the figure. Cells were then washed in 37°C buffer and 37°C growth media containing 200 nM LTR was added for 30 minutes. Cell samples were lysed and measured for cell associated fluorescence and normalized to cellular protein content. Cells treated with imipramine at 37°C showed a significant increase in the accumulation of LTR, whereas, imipramine treatment at 4°C failed to show a change in the ion trapping capacity of cells as measured by LTR accumulation. Cellular accumulation of LTR as a percent of the vehicle-treated control cells is represented as mean \pm S.D of six values for each sample (***, $p < .001$ by Student's t test).

3.3.6. Calculation of the change in apparent lysosomal volume

The cellular accumulation of weakly basic compounds, like LTR, by ion trapping in lysosomes is predicted to depend on two important parameters: 1) lysosomal pH and 2) lysosomal volume. Approximations of the impact of changes in either parameter can be predicted by simple steady state equations derived in the original work describing lysosomal ion trapping (de Duve, de Barse et al. 1974). These equations (see *Materials and methods*, Section 3.2.7) simply illustrate that a change in the lysosomal pH (i.e., hydrogen ion concentration) or lysosomal volume results in proportional changes in cellular uptake by lysosomal ion trapping. In this work, the effect of imipramine on the cellular accumulation of LTR by lysosomal ion trapping (Figure 3.6) and lysosomal pH were measured. Therefore, these experimental values can be used to calculate the change in lysosomal volume following imipramine treatment. Using the data from Figure 3.6, the amount of LTR accumulated in cells following ionophore treatment was subtracted from the amount without ionophore treatment and yielded the amount of LTR accumulating in cells by lysosomal ion trapping (Figure 3.16). Imipramine treatment caused an approximately three-fold increase in lysosomal ion trapping-based accumulation of LTR. In conjunction with the measured lysosomal pH values in imipramine treated and vehicle-treated cells an approximately three- to four-fold increase in the apparent lysosomal volume following imipramine treatment was calculated. Because other acidic organelles, such as the Golgi apparatus and late endosomes, may contribute to LTR accumulation the calculated volume change is denoted as a change in the apparent lysosomal volume. These observations were

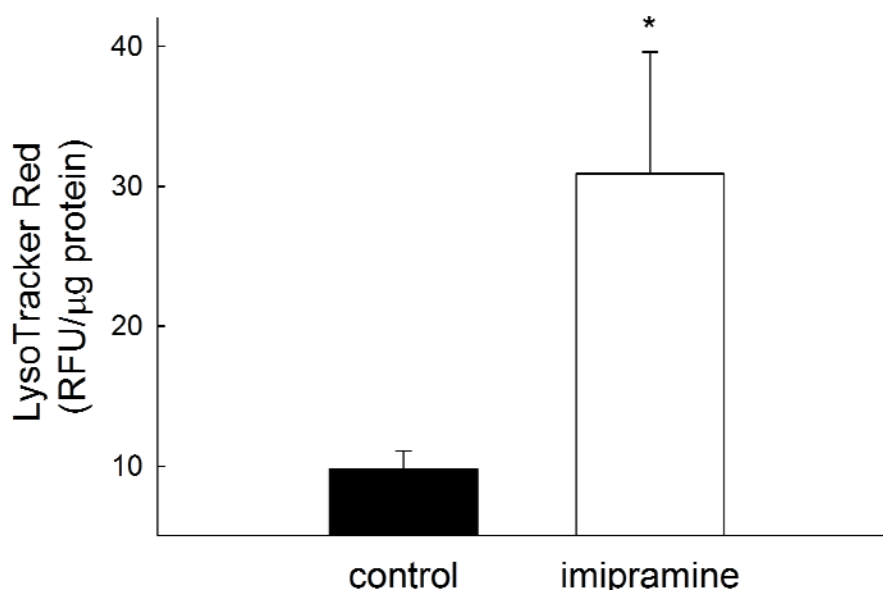


Figure 3.16. Measurement of increased ion trapping capacity following CAD treatment. The amount of the model substrate for lysosomal ion trapping, LTR, accumulated in cells by ion trapping-based accumulation was measured in MDA-1986 cells that were treated with 10 μ M imipramine (white bar) or vehicle alone (black bar) for 24 hours. In the last 2 hours of treatment, 200 nM LTR was added and cells were either co-treated with ionophores (10 μ M nigericin and 20 μ M monensin) to eliminate intracellular pH gradients or left untreated to retain an intact lysosome/cytosol pH gradient. Cells were subsequently lysed and LTR accumulation was assessed by measuring cell-associated fluorescence signal. Fluorescence signal was normalized to cellular protein content. The amount of LTR accumulated in ionophore treated cells was subtracted from the amount accumulated in cells under normal cell growth conditions resulting in the amount of LTR accumulated in cells by lysosomal ion trapping, for both vehicle-treated and imipramine treated cells. Imipramine treatment resulted in a significant increase in the amount of LTR accumulated in cells by ion trapping. Cellular accumulation of LTR by lysosomal ion trapping is represented in fluorescence counts per μ g of protein as mean \pm S.D of three values for each sample (*, $p < 0.05$ by Student's t test).

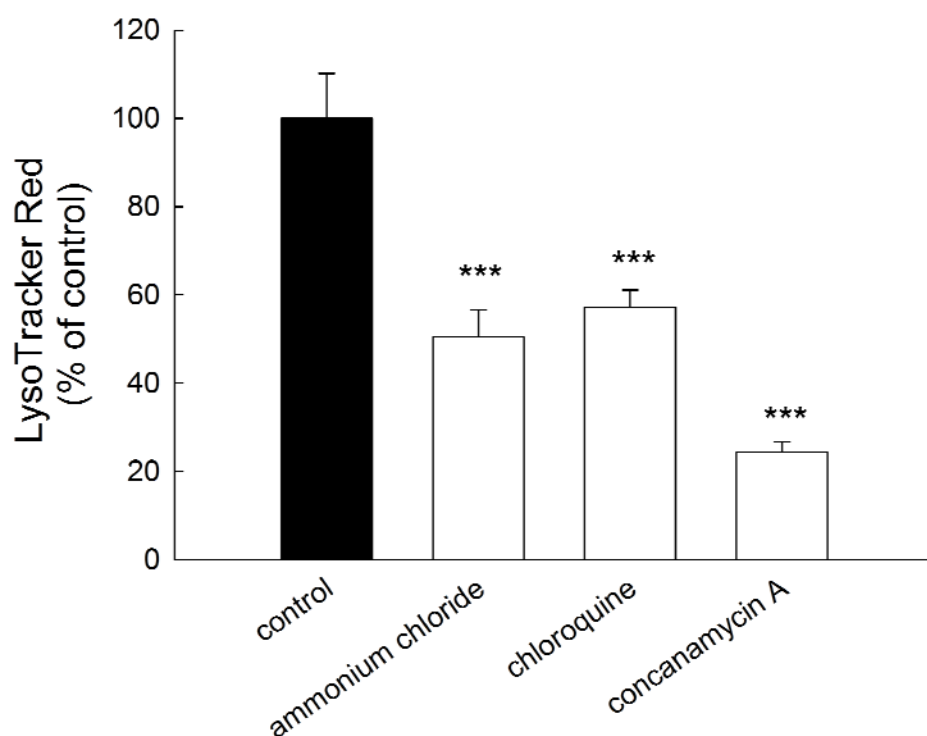
consistent with the microscopy studies presented in Chapter 2 that suggested an increase in the vesicular accumulation in an expanded lysosomal compartment.

The results here are in agreement with recent work that has shown a progressive, non-steady state accumulation profile for the phospholipidosis-inducing drug chloroquine (Zheng, Zhang et al. 2010). Similar to the results presented here, these authors found that chloroquine induced the formation of an expanded acidic vesicular compartment that resulted in enhanced cellular accumulation of the phospholipidosis-inducing drug. Their data shows a 10- to 30-fold increase in vesicular volume with up to a one-unit upward shift in the vesicular pH. Although the CAD treatments here showed a less pronounced increase in apparent lysosomal volume and no significant increase in vesicular pH at the concentration of imipramine used, it is believed that similar effects would be observed with increasing drug concentrations. The differences in vesicular volume measurements may also originate from their microscopy-based measurements of vesicular volume and the calculation-based method used in this study. Additionally, these authors found that inhibition of the phospholipidosis phenotype by treatment with bafilomycin A1 reversed the expansion of the vesicular volume in agreement with the results presented here. Therefore, in addition to the previously established capability of phospholipidosis-inducing drugs to increase their own cellular accumulation in an expanded acidic vesicular compartment, it was shown here that they also increase the cellular accumulation of other drugs that accumulate in these acidic subcellular compartments.

3.3.7. Dose dependent effect of CADs on LTR accumulation suggests lysosomal alkalinization with increased dose

Previous work has illustrated the possibility that amine-containing drugs, such as CADs, accumulating in the lysosome can cause alkalinization of the lysosome and inhibit lysosomal ion trapping of secondarily administered amine-containing compounds (Daniel 2003), such as LTR. The current studies confirmed these findings by measuring LTR accumulation in cells exposed to either 30 μ M chloroquine, 10 mM ammonium chloride or 200 nM of the vacuolar-type H^+ -ATPase inhibitor concanamycin A (Figure 3.17). All three of these well-established lysosomal alkalinizing treatments significantly decreased the cellular accumulation of LTR. Therefore, LTR accumulation in cells treated with a range of imipramine doses between 0.01 and 50 μ M was evaluated (Figure 3.18). Imipramine appeared to cause a dose-dependent effect, whereby, LTR accumulation increased with doses up to 1 μ M. Doses above 1 μ M caused a gradual decrease in LTR accumulation.

These results would be in agreement with the hypothesis that CAD treatment has multiple dose-dependent effects on lysosomes that influence their ability to accumulate substrates for lysosomal ion trapping. Perhaps at lower, non-alkalinizing doses, an expansion of the lysosomal volume causes increased lysosomal ion trapping, but as the dose increases, the CAD begins to cause lysosomal alkalinization and reduces lysosomal ion trapping. Whatever the reason for the apparent dose-response, it shows that the more therapeutically relevant lower imipramine doses cause an increased capacity to accumulate substrates for lysosomal ion trapping that is reversed at increased supratherapeutic doses.



*Figure 3.17. Alkalinization of lysosomes decreases the cellular accumulation of the model substrate for lysosomal ion trapping, LTR. MDA-1986 cells grown under normal cell culture conditions were treated for 2 hours with vehicle alone (black bar) or various treatments previously shown to cause lysosomal alkalinization (white bars), including 30 μ M chloroquine, 10 mM ammonium chloride or 200 nM concanamycin A. LTR at a concentration of 200 nM was added concomitant to the various treatments. At the end of the 2-hour treatment cells were lysed and measured for cellular accumulation of LTR by measuring cell associated fluorescence signal. Fluorescence counts were normalized to cellular protein content. Cellular accumulation of LTR was normalized to percent vehicle-treated control. All lysosomal alkalinizing treatments significantly reduced the lysosomal ion trapping capacity of cells as indicated by the decreased cellular accumulation of LTR. Cellular accumulation of LTR as a percent of the vehicle treated control cells is represented as mean \pm S.D of at least three values for each sample (***, $p < .001$ by Student's t test).*

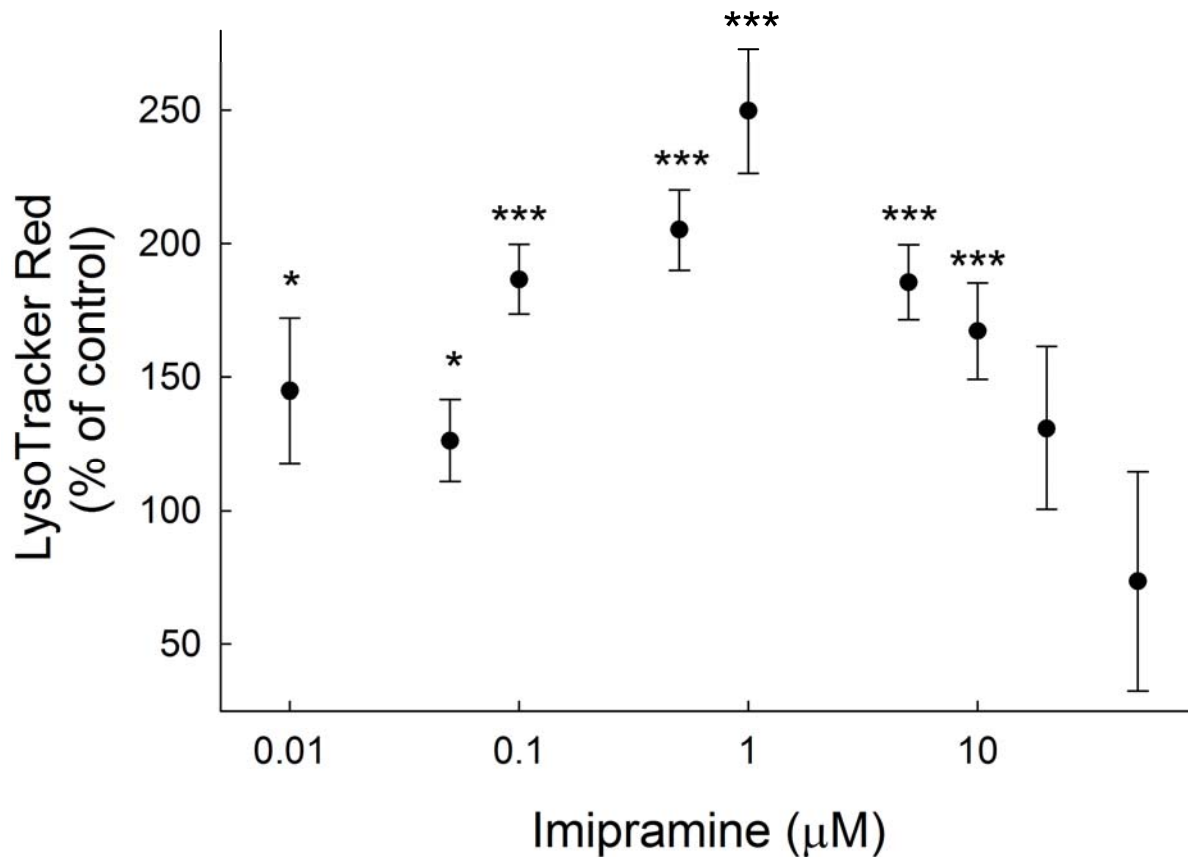


Figure 3.18. Cellular accumulation of the model substrate for lysosomal ion trapping, LTR, following CAD treatment is dose-dependent. Fibroblasts were treated for 24 hours with a range of imipramine concentrations between 0 and 50 μM imipramine. In the last 2 hours, 200 nM LTR was added. Samples were subsequently lysed and measured for cell-associated fluorescence. Fluorescence signal was normalized to the cellular protein content of each sample. Cellular accumulation of LTR was normalized to percent vehicle-treated control (i.e., 0 μM imipramine). Imipramine, at doses below 1 μM caused a dose-dependent increase in the cellular accumulation of LTR. At concentrations above 1 μM, this trend is reversed. Cellular accumulation of LTR as a percent of the vehicle-treated control cells is represented as mean ± S.D of at least three values for each sample (***, $p < .001$ by Student's t test).

3.4. Conclusions

Collectively, this work illustrates a novel intracellular drug-drug interaction pathway resulting from the ability of CADs to cause an expansion of the apparent lysosomal volume of cells. This lysosomal volume expansion was found to be secondary to the CAD-induced cellular lipidosis and to result in the increased cellular uptake of drugs that are substrates for ion trapping-based accumulation in lysosomes. A change in the cellular uptake and accumulation of these drugs reveals a novel distribution-based drug-drug interaction pathway with clinically relevant implications that necessitates further in vivo studies.

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Chapter 4

Drug-induced lysosomal volume expansion fails to cause a generalized decrease in lysosomal enzyme activity

4.1. Introduction

In this work it is hypothesized that the drug-induced lysosomal volume expansion results in the apparent dilution of enzymes within the lysosomal compartment and results in a generalized decrease in lysosomal hydrolase activity that causes the lysosomal accumulation of undigested lipids (Lullmann, Lullmann-Rauch et al. 1973) and other lysosomal cargoes (Kaufmann and Krise 2008) that is often observed following CAD treatment. Such findings would be important in understanding the biochemical mechanism through which CADs inhibit the normal metabolism of lipids that results in the toxicological phenomenon often referred to as phospholipidosis. Therefore, this chapter assesses lysosomal hydrolase activity in cells with an expanded lysosomal compartment as a possible mechanism of a general reduction in the metabolism of substrates for lysosomal enzymes.

Lysosomes play an important role in the cellular metabolism of numerous substrates, including: lipids, carbohydrates, nucleic acids and proteins. The digestion of these substrates within lysosomes occurs primarily via enzymatic hydrolysis that is favored by the acidic environment of the lysosome (Sun-Wada, Wada et al. 2003). Changes in the properties of lysosomes have been shown to effect lysosomal hydrolase activity, such as lysosomal alkalization (Ohkuma, Chudzik et al. 1986). Interestingly, in Chapter 3 it was found that hydrophobic amine-containing drugs, commonly referred to as cationic amphiphilic drugs (CADs), can alter lysosomes by causing an expansion of the lysosomal compartment without significantly affecting lysosomal pH. The expansion of the lysosomal compartment appears to be related to the concomitant

lipidosis caused by these drugs and may indicate a relationship between the two events.

Lysosomal enzymes are synthesized in the endoplasmic reticulum and transported to the Golgi apparatus where the majority of lysosomal proteins undergo posttranslational tagging with phosphomannosyl resulting in binding to mannose-6-phosphate receptors (M6PR) with subsequent transport to lysosomes. Entry into the acidified endosomal/lysosomal compartments causes the receptor-enzyme complex to dissociate. Within the lysosome the enzyme often enters as an inactive pro-form that requires further activation by other lysosomal enzymes (Kornfeld 1986). In addition, the acidic pH optimum for activity of these enzymes allows them to be highly active within the acidified lysosome (Coffey and De Duve 1968). Genetic defects resulting in defective synthesis, trafficking, processing or catalytic activity of these enzymes can result in one of a number of lysosomal storage diseases (Kornfeld 1986). Similarly, drugs that affect any of these processes would also be predicted to affect lysosomal enzyme activity. Drugs that cause lysosomal alkalinization resulting in inhibition of lysosomal hydrolase activity (Ohkuma, Chudzik et al. 1986) would fit this category. A group of hydrophobic amine-containing drugs that are often referred to as CADs are well known to inhibit the normal lysosomal digestion of lipids resulting in a phenotype similar to that observed in many lysosomal storage diseases (Schneider, Korolenko et al. 1997). Although the mechanism through which these drugs inhibit lysosomal lipid metabolism remains unclear it has been observed that these drugs, for the most part, fail to directly inhibit enzyme-mediated hydrolysis of lipids and other lysosomal enzyme substrates in cellular homogenate (Yoshida, Arimoto et al. 1985). The necessity of a

physiologically intact cellular system for these drugs to inhibit lysosomal lipid metabolism suggests that these drugs inhibit lysosomal lipid metabolism through an indirect pathway. The finding in Chapter 3 that CADs cause an expansion of the lysosomal compartment led to the hypothesis that CADs may actually inhibit lysosomal lipid metabolism through the dilution of lysosomal enzymes that results from an expanded lysosomal compartment. It was reasoned that changes in the absolute concentration of the enzymes within the lysosome could result in a possible reduction in enzyme activity, and therefore, the accumulation of enzyme substrates within the lysosome. These substrates would not only include lipids, but also carbohydrates, nucleic acids and proteins.

In this chapter, the effect of the expanded lysosomal compartment, following CAD treatment, is investigated as a mechanism of reduced lysosomal hydrolase activity. Following treatment with the CAD, imipramine, the lysosomal hydrolase activity as determined by the proteolytic degradation of a fluorescently labeled protein, DQ-BSA, is measured. Similarly, the activity of the lysosomal hydrolase, cathepsin B, is measured using a synthetic substrate specific for the enzyme.

4.2. Materials and methods

4.2.1. Cell lines and reagents

DQ-BSA, Dulbecco's phosphate buffered saline (D-PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Daunorubicin was purchased from Oakwood Products (West Columbia, SC). Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-R-R-NHMec) and imipramine were

purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). MDA-1986 squamous cell carcinoma cells from the laboratory of Dr. M.S. Cohen at the University of Kansas Medical Center were maintained in DMEM supplemented with 10% FBS and maintained at 37°C and 5% CO₂, all experiments were carried out under these conditions unless otherwise stated. Cells were routinely subcultured to maintain 60% to 80% confluency.

4.2.2. DQ-BSA proteolytic activity assay

MDA-1986 cells were seeded onto 96-well plates at 5×10^3 cells/well in growth media containing DQ-BSA at concentrations up to 100 µg/ml. Time-based measurements of fluorescence signal were obtained using a Bio-Tek FL600 microplate fluorescence reader with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Good signal to noise and a linear increase in fluorescence was observed with 100 µg/ml DQ-BSA at 48 hours. Therefore, MDA-1986 cells were subsequently seeded in a 96-well plate with 100 µg/ml DQ-BSA for 48 hours without drug addition (control) or along with 10 µM imipramine. The cell-associated fluorescence signal of each sample was measured. The cells were subsequently lysed by the addition of 0.1 M NaOH and cellular protein content was determined by the BCA method. Fluorescence signal was normalized to sample protein content. Proteolytic activity was expressed as fluorescence per microgram of cell protein.

4.2.3. Cellular cathepsin B activity assay

MDA-1986 cells were seeded in a 96-well plate at 1×10^4 cells/well and allowed to adhere. Samples were either left untreated (control) or treated with 10 μ M imipramine for 24 hours. The cathepsin B substrate, Z-R-R-NHMec was added to the cells in order to attain concentrations up to 125 μ M and cells were maintained at 37°C. Immediately upon addition of the substrate, time-based fluorescence measurements were obtained using a Bio-Tek FL600 microplate fluorescence reader with excitation and emission wavelengths set at 360 nm and 460 nm, respectively. Kinetic plots of fluorescence versus time for each substrate concentration were constructed for control and imipramine treated cells. Initial rates of product formation determined by the slope of the early linear region of each plot was termed the “Reaction Velocity” in RFU per minute. A Michaelis-Menten plot of the reaction velocity as a function of substrate concentration was constructed for control and imipramine-treated cells. The data were fit by non-linear regression to the Michaelis-Menten equation using SigmaPlot (SPSS Inc., Chicago, IL) from which the constants V_{max} and K_m were obtained.

4.3. Results and discussion

4.3.1. Lysosomal volume expansion and cellular protein degradation

Exogenously supplied bovine serum albumin (BSA) has been used as a marker for the lysosomal hydrolase mediated proteolysis of endocytosed proteins (Ohkuma, Chudzik et al. 1986). The ability of basic substances and ionophores to inhibit the digestion of BSA has been proposed to result from the lysosomal alkalinizing effect of these compounds. Therefore the digestion of BSA may serve as a marker for the

lysosomal hydrolase activity of cells. The first objective was to determine whether expansion of the lysosomal compartment by CAD treatment would affect the degradation of exogenously supplied BSA. It was reasoned that a decrease in BSA degradation would be indicative of a general reduction in lysosomal hydrolase activity. To measure BSA degradation a highly fluorescently tagged form of the protein, known as DQ-BSA, was employed. The high degree of fluorescent labeling results in a low level of fluorescence due to self-quenching, but upon proteolytic degradation the fluorophores become un-quenched and a relative increase in fluorescence is observed (Figure 4.1). Cells were treated with the CAD, imipramine, at the dose and duration illustrated as causing an expansion of the lysosomal compartment in Chapter 3. In addition, DQ-BSA was added for the 48-hour treatment. CAD treatment resulted in no apparent change in the level of proteolytic degradation of DQ-BSA, as measured by the cell-associated fluorescence (Figure 4.2). Contrary to the hypothesis, expansion of the lysosomal compartment by CADs did not result in a generalized reduction in lysosomal hydrolase activity as measured by the proteolytic digestion of BSA. Although, DQ-BSA has been suggested as having both lysosomal and non-lysosomal degradation pathways (Sameni, Moin et al. 2000), and therefore, it may be conceivable that non-lysosomal-mediated degradation may be compensatory in the CAD treated cells, or non-lysosomal degradation may be the primary route of digestion. Therefore, specific measurement of lysosomal hydrolase activity in living cells may provide a clearer picture of the impact of lysosomal volume expansion on lysosomal hydrolase activity.

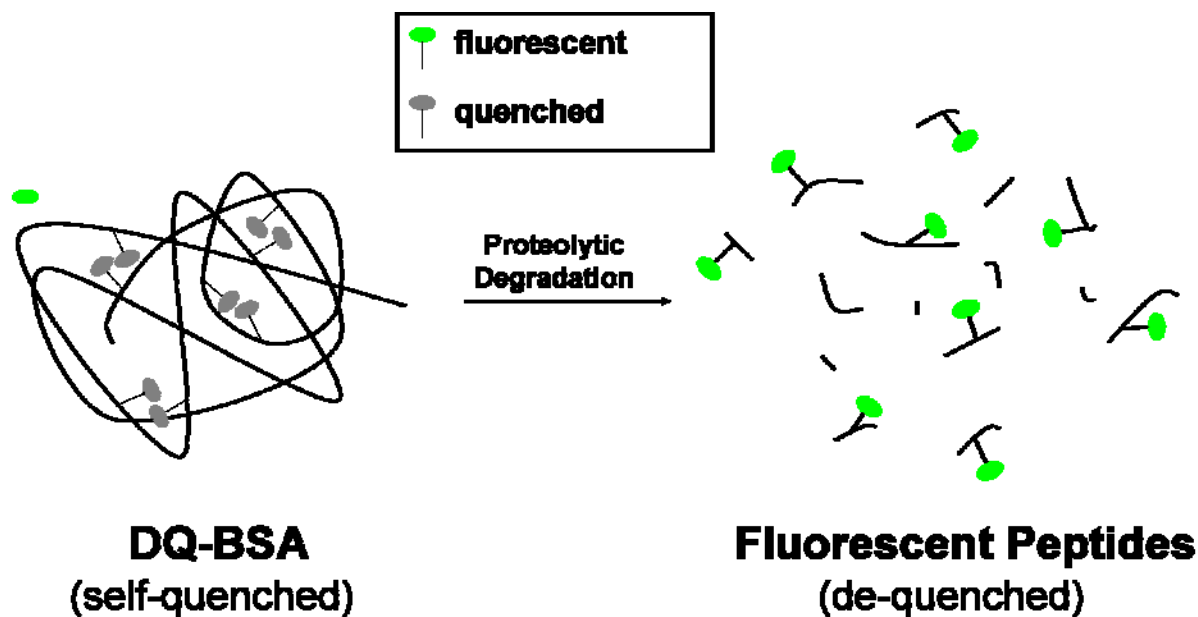


Figure 4.1. DQ-BSA proteolytic activity assay. Fluorescently labeled bovine serum albumin (DQ-BSA) is highly self-quenched yielding low fluorescence. Upon proteolytic degradation of DQ-BSA to fluorophore-labeled peptides the reduced self-quenching results in increased fluorescence. Generation of fluorescence signal provides a quantitative measurement of proteolytic activity.

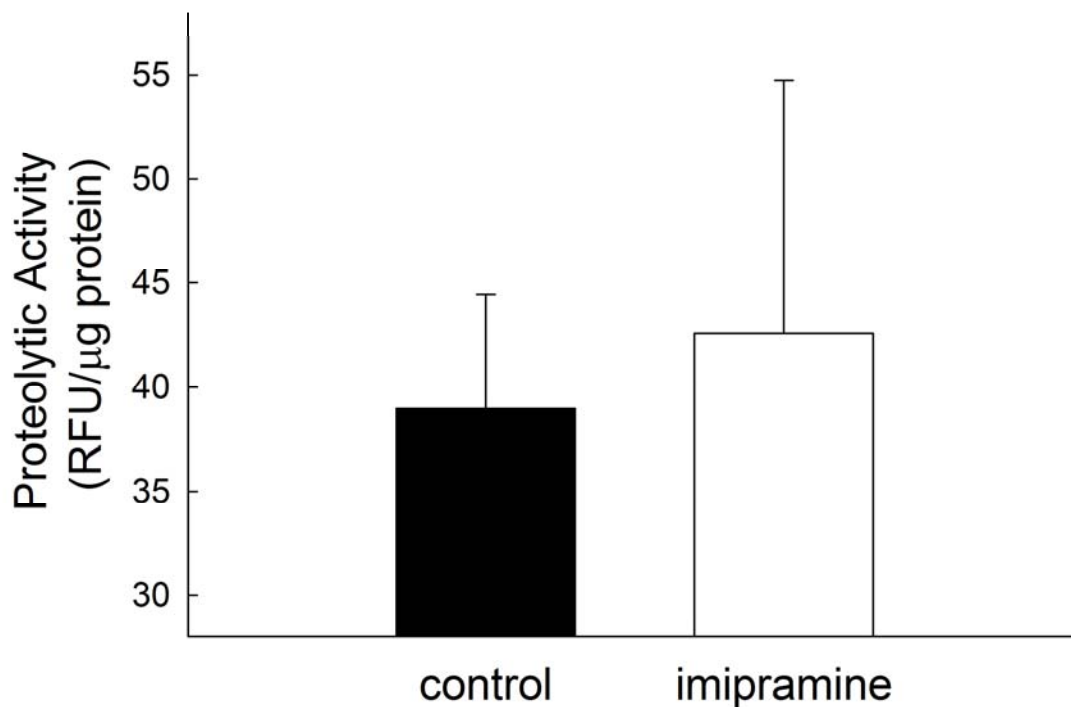


Figure 4.2. Effect of CAD-induced lysosomal volume expansion on proteolytic activity. MDA-1986 cells were left untreated (control) or treated with 10 μ M imipramine for 48 hours in the presence of 100 μ g/ml DQ-BSA. Proteolytic activity was determined by measuring cell-associated fluorescence. The CAD, imipramine, caused no significant change in proteolytic activity. Cell-associated fluorescence was normalized to cellular protein content and proteolytic activity is represented as mean \pm S.D. of at least three values for each sample.

4.3.2. Lysosomal volume expansion and cathepsin B activity

A water-soluble substrate for cathepsin B has been previously used to measure lysosomal enzyme activity in living cells (Ulbricht, Spiess et al. 1995). The non-fluorescent enzyme substrate, Z-R-R-NHMec is hydrolyzed by cathepsin B to form the fluorescent product, 7-amino-4-methylcoumarin, which allows measurement of enzyme activity using a simple fluorescence plate reader-based assay (Figure 4.3). The specificity of this substrate for cathepsin B allows for the measurement of lysosomal hydrolase activity in live cells, presumably without contribution from extralysosomal sources of hydrolysis. Judging by the structure of the probe, it would not be expected to be membrane permeable based on the two arginine residues that would be positively charged at physiological pH, but it and similar cathepsin B substrates have been shown to be rapidly hydrolyzed (Ulbricht, Spiess et al. 1995) and localized to intracellular vesicular compartments that are presumed to be lysosomes (Premzl, Turk et al. 2006), suggesting that it rapidly accesses the lysosomal compartment. The cellular activity of cathepsin B was measured by applying the non-fluorescent substrate Z-R-R-NHMec to cells and measuring the formation of the fluorescent hydrolysis product. Following a 24-hour treatment with vehicle alone or the lysosomal volume expanding CAD, imipramine, concentrations of the Z-R-R-NHMec between 5 μ M and 125 μ M were added to cells and fluorescence signal was measured as a function of time. By measuring the initial rate of product formation by a linear fit of the early timepoints, the reaction velocities were determined in RFU/min and plotted for vehicle treated (control) and imipramine treated cells (Figure 4.4). As seen by the plot, the lysosomal volume expanding CAD treatment didn't appear to significantly affect the reaction velocities at any of the substrate

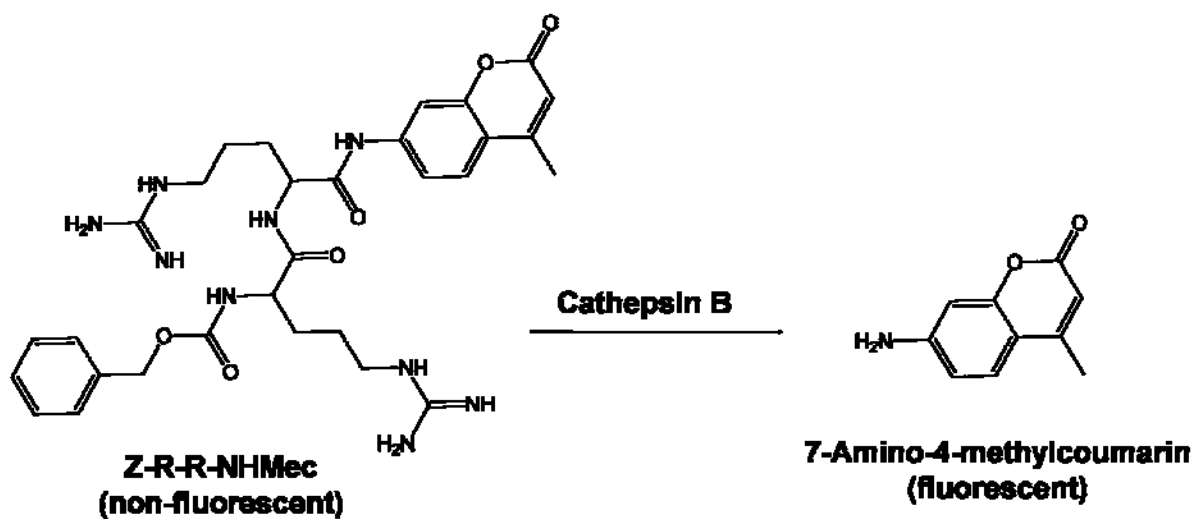


Figure 4.3. Cathepsin B activity assay. The non-fluorescent substrate Z-R-R-NHMec is hydrolyzed by the lysosomal hydrolase cathepsin B to form a fluorescent product, 7-amino-4-methylcoumarin. Generation of the fluorescent product allows enzyme activity measurements in biological systems by measuring the change in sample fluorescence as a function of time.

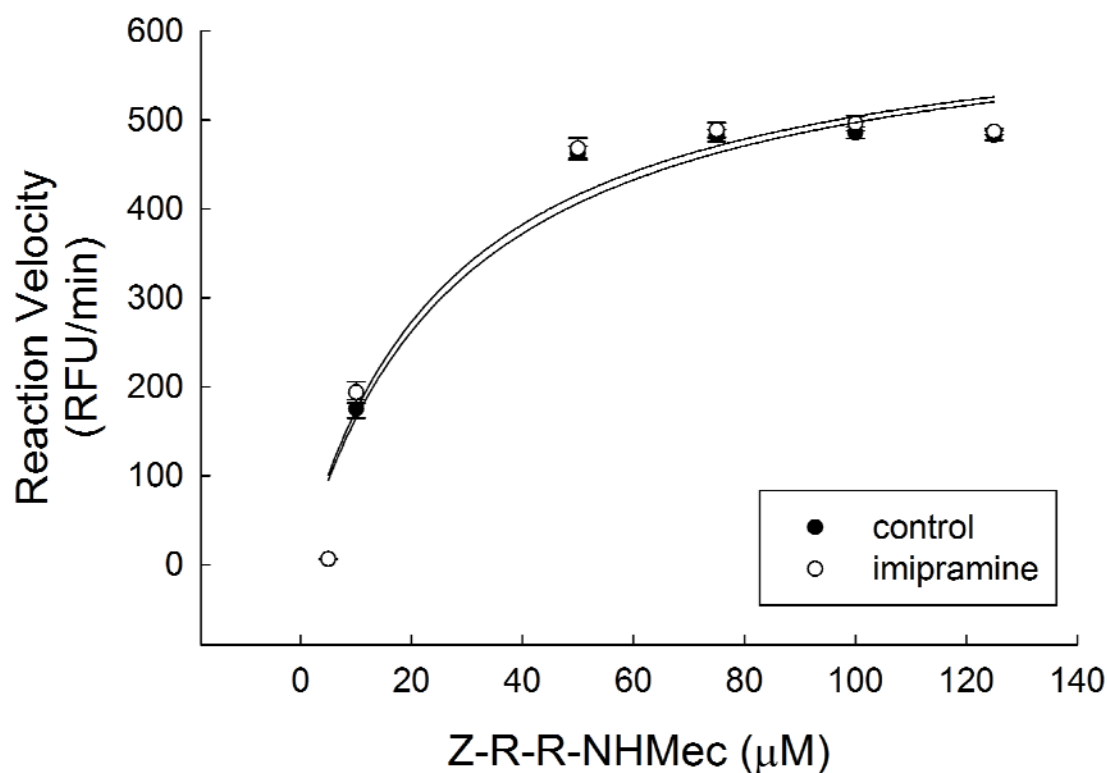


Figure 4.4. Effect of CAD-induced lysosomal volume expansion on cellular cathepsin B activity. MDA-1986 cells were left untreated (control) or treated with 10 μM imipramine for 24 hours. Z-R-R-NHMec was added at concentrations between 5 μM and 125 μM . Formation of the Z-R-R-NHMec hydrolysis product, 7-amino-4-methylcoumarin, was measured by time-based fluorescence reading and the initial rates were determined and defined as reaction velocities in RFU/min. A plot of reaction velocities versus substrate concentration is presented. Cathepsin B activity is represented as mean \pm S.D. of at least three values for each sample. Data were fit with a non-linear regression to the Michaelis-Menten equation using SigmaPlot, control curve ($R^2 = 0.9354$) and imipramine curve ($R^2 = 0.9391$). K_m and V_{max} were determined from the triplicate measurements and expressed as a mean \pm S.D.

concentrations tested. The triplicate measurements were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} . The V_{max} for control and imipramine treated cells were 640.5 ± 13.0 RFU/min and 639.0 ± 5.3 RFU/min, respectively. The K_m for control and imipramine treated cells were determined to be 28.85 ± 1.22 μ M and 26.87 ± 1.35 μ M, respectively. Therefore, no appreciable changes in the kinetic parameters of cathepsin B were observed following imipramine treatment. These results further illustrate that the lysosomal volume expansion caused by CAD treatment doesn't result in a general reduction in the lysosomal hydrolase activity of cells.

4.4. Conclusions

This work sought to describe the effect of lysosomal volume expansion on lysosomal hydrolase activity as a potential mechanism of indirect inhibition of lysosomal lipid metabolism following CAD treatment. The hypothesis that lysosomal volume expansion could result in reduced enzymatic activity was not supported by the results. Because CADs cause both a lysosomal volume expansion and an increase in the lysosomal accumulation of undigested lipids, it was hypothesized that the lysosomal volume expansion may result in a generalized reduction in lysosomal hydrolase activity that decreases the metabolism of lipids and other lysosomal enzyme substrates. This hypothesis was based on previous observations that CADs fail to directly inhibit lysosomal lipid hydrolase activity, but rather require an intact cellular system in order to mediate their activity. Although, this indirect inhibition could result through a variety of mechanism, the observation of a drug-induced expansion of lysosomes warranted further investigation as a possible mechanism. By treating with the lysosomal volume

expanding CAD, imipramine, it was found that cells showed no change in either proteolytic digestion of the fluorescently labeled protein, DQ-BSA, or lysosome-specific hydrolase activity as measured using the cathepsin B substrate, Z-R-R-NHMec.

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Chapter 5

Lysosomal volume expansion and alkalinization caused by amine-containing drugs

5.1. Introduction

In this chapter, the effect of hydrophobic ($\log P > 2.5$) and hydrophilic ($\log P < 2.5$) amine-containing compounds on the structural and functional properties of lysosomes related to the lysosomal ion trapping capacity of cells is evaluated. The lysosomal ion trapping-based accumulation of amine-containing drugs is well established as causing a number of dose-dependent functional and structural perturbations in lysosomes, including: alkalinization of the normally acidic lysosome (Ohkuma and Poole 1978), osmotic swelling of lysosomes (Ohkuma and Poole 1981; Morissette, Lodge et al. 2008) and the lysosomal accumulation of undigested lipids (Halliwell 1997). In chapters 2 and 3, exposure to hydrophobic amine-containing drugs, known as CADs, were found to cause an apparent expansion of the lysosomal compartment that results in the increased cellular accumulation of substrates for ion trapping in lysosomes. Interestingly, previous studies have also illustrated the ability of amine-containing drugs to cause an apparent expansion of the aqueous volume of lysosomes resulting in a cytoplasmic vacuolization (Morissette, Moreau et al. 2004). In this chapter it is hypothesized that the cytoplasmic vacuolization caused by some amine-containing drugs is distinct from the CAD-induced lysosomal lipidosis and that the cytoplasmic vacuolization is accompanied by a concomitant alkalinization of lysosomes that results in a failure to cause the increase in lysosomal ion trapping capacity observed with the lysosomal lipidosis following CAD treatment.

Weakly basic amine-drugs accumulate in lysosomes through an ion trapping-type mechanism that is driven by the pH gradient existing between the acidic lysosome and the extralysosomal environment (de Duve, de Barsey et al. 1974). Therefore, the amount

of drug accumulated in cells by ion trapping in lysosomes is dependent on the pH gradient existing between the lysosome and the extralysosomal space, as well as, the volume of the lysosomal compartment. A change in either the lysosomal volume or pH is presumed to result in significant deviations in the lysosomal ion trapping capacity of cells. The alkalinization of lysosomes by exposure to high concentrations of amine-drugs would be expected to decrease the lysosomal ion trapping capacity of cells, whereas the expansion of the lysosomal compartment in cytoplasmic vacuolization and lysosomal lipidosis would be expected to increase the lysosomal ion trapping capacity. Therefore, the interplay between lysosomal alkalinization and expansion of the lysosomal compartment caused by these drugs is important in predicting the effect they will have on the lysosomal ion trapping capacity of cells. Such an ability of one compound to alter the cellular disposition of another has been predicted here (see chapter 3), as well as by others (Daniel and Wojcikowski 1999), to represent the potential for distribution-based drug-drug interactions.

The proposed mechanisms whereby amine-containing drugs cause lysosomal alkalinization, cytoplasmic vacuolization and lysosomal lipid accumulation would suggest that these effects occur through distinct molecular mechanisms. Empirical evidence suggests that hydrophobicity (i.e., logP) is a key predictor of the ability and potency of these compounds in causing these effects on the lysosome. Hydrophobicity has been found to be directly related to potency in causing alkalinization in isolated lysosomes (Ishizaki, Yokogawa et al. 2000). Similarly, the lysosomal lipidosis has been strongly correlated with amine-containing drugs of significant hydrophobicity (logP > 2.5) (Lullmann-Rauch 1979) and cytoplasmic vacuolization is primarily observed with the

less hydrophobic amine-containing compounds (Ohkuma and Poole 1981). Such observations suggest a potential dichotomy amongst amine-containing compounds where the hydrophobicity of the drug is a key predictor of its effect on lysosomes. Therefore, the ability of amine-containing drugs, based on their relative hydrophobicity, to affect key lysosomal properties, such as pH and volume, suggests that hydrophobicity may also be an important predictor in the ability of these compounds to affect the lysosomal ion trapping capacity of cells.

Here, the model hydrophilic amine, ammonium chloride, and the model hydrophobic amine, imipramine, are evaluated for their ability to cause cytoplasmic vacuolization, lysosomal lipid accumulation and lysosomal alkalinization. It is shown that these compounds differ in their potency and capability in altering the functional and structural properties of lysosomes. Although, ammonium chloride causes an apparent expansion of the aqueous volume of lysosomes (i.e., cytoplasmic vacuolization) it fails to cause an increase in the lysosomal ion trapping capacity of cells. The failure to cause an increase in lysosomal ion trapping is attributed to the concomitant alkalinization of lysosomes that occurs at the high doses necessary to cause the cytoplasmic vacuolization. These results support the hypothesis that hydrophobic and hydrophilic amine-containing compounds differ in their potency and ability in altering the properties of lysosomes.

5.2. Materials and methods

5.2.1. Cell lines and reagents

NBD-PE, LysoTracker Red DND-99 (LTR), Dulbecco's phosphate buffered saline (D-PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Propranol, procainamide, Nile red, mannitol, methylamine, imipramine, ammonium chloride, neutral red, morpholine, imidazole, isopropylamine, diisopropylamine, U18666A, amiodarone, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). CRL-2076 human foreskin fibroblasts and HeLa cervical adenocarcinoma cells purchased from ATCC (Manassas, VA) were maintained in DMEM supplemented with 10% FBS and maintained at 37°C and 5% CO₂, all experiments were carried out under these conditions unless otherwise stated. Cells were routinely subcultured to maintain 60% to 80% confluency.

5.2.2. Neutral red uptake inhibition assay

Fibroblasts seeded at 1×10^4 cells/well in 96-well plates were allowed to adhere overnight under normal growth conditions. Media was replaced with fresh media containing titrated drug concentrations in triplicate. Cells were incubated for 1 hour under normal growth conditions followed by the addition of 10 µM neutral red for an additional 1 hour. Upon removal of media each well was washed once with 4°C D-PBS and replaced with a destain solution containing 50% ddH₂O, 49% ethanol and 1% glacial acetic acid. Fluorescence was measured using a Bio-Tek FL600 microplate fluorescence reader with excitation and emission wavelengths set at 530 nm and 645

nm, respectively. Background signal from neutral red treated wells without cells was subtracted from each reading and untreated control cells were used to normalize fluorescence readings to calculate “Neutral Red Uptake Inhibition (% of control)” that was plotted as a function of drug concentration on a log scale. The resulting data were fitted using a 3- or 4-parameter Hill equation in SigmaPlot (SPSS Inc., Chicago, IL) and the concentration of drug necessary to cause a 50% inhibition of neutral red uptake (i.e., IC_{50}) was calculated. A Pearson’s correlation of the log of the IC_{50} versus logP was conducted using Wessa statistics software (free online at www.wessa.net).

5.2.3. WST-1 cell viability assay

Cell viability in imipramine treated HeLa cells was measured using the WST-1 cell viability assay according to the manufacturer’s protocol (Roche Molecular Biochemicals, Mannheim, Germany). The WST-1 assay measures mitochondrial dehydrogenase activity through the formation of a reduction product with an absorbance at 450 nm. Briefly, HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and allowed to adhere overnight under normal growth conditions. Media was removed and replaced with media containing imipramine at concentrations up to 200 μ M for 24 hours. WST-1 reagent was added to each sample and maintained under normal growth conditions for 3 hours. A microplate absorbance reader was used to measure sample absorbance at 450 nm. Untreated control cells served as the 100% viability reference from which ‘Dehydrogenase Activity’ was determined and plotted against imipramine concentration on a log scale. The data was fit to a 4-parameter Hill

equation using SigmaPlot (SPSS Inc., Chicago, IL) that was used to calculate the concentration necessary to cause 50% inhibition of dehydrogenase activity (IC_{50}).

5.2.4. NBD-PE accumulation

HeLa cells were seeded onto a 24-well plate at 2.5×10^4 cells/well in growth media containing 50 μ M NBD-PE with or without concomitant drug treatment following a previously established protocol (Fujimura, Dekura et al. 2007) with slight modifications. Cells were maintained under normal growth conditions for 96 hours. Cells were washed twice with warm D-PBS and lysed with 0.1 M NaOH. Lysate fluorescence was measured using a Bio-Tek FL600 microplate fluorescence reader with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Cell lysate was measured for protein content using the BCA method and fluorescence signal was normalized to protein content. NBD-PE accumulation was expressed as the mean and standard deviation from triplicate measurements.

5.2.5. Cell microscopy studies

Fibroblasts grown on chambered glass microscope slides were exposed to 70 μ M neutral red for 6 hours followed by a brief washing in 37°C D-PBS and a 2 hour chase in fresh 37°C media with or without 200 nM concanamycin A. Cells were subsequently washed once with 37°C D-PBS and sealed with a coverslip for imaging. Nile red staining required the addition of 2 μ g/ml Nile red for 30 minutes under normal growth conditions and cells were washed twice with room temperature D-PBS prior to

imaging. Vacuolization studies utilized drug treatment times between 2 hours and 24 hours and cells were either imaged alive or after fixing with 4% paraformaldehyde.

The cells were viewed using a Nikon Eclipse 80i or Eclipse TE2000-U microscope equipped with either a 40x (1.30 NA) or 60x (1.40) oil immersion objective for fluorescence or DIC microscopy. Fluorescence images were acquired using the filter cube that best overlapped with the documented spectral properties of each fluorophore. Samples for fluorescence imaging were excited with an EXFO X-Cite 120 PC fluorescence illumination system. Images were acquired using an ORCA ER camera (Hamamatsu). Images were analyzed using Metamorph version 7.0 (Universal Imaging) and/or ImageJ (free online at rsbweb.nih.gov) software. Background images of cells not exposed to fluorescent probe were acquired to correct for autofluorescence and images were scaled identically to allow for comparison.

5.3. Results and discussion

5.3.1. Effect of hydrophilic amine-containing drug treatment on the lysosomal ion trapping capacity of cells

In chapter 3 the hydrophobic amine, imipramine was shown to increase the lysosomal ion trapping capacity of cells by causing an increase in the volume of the lysosomal compartment. Although, increased imipramine concentrations resulted in increased lysosomal ion trapping capacity up to a point, at higher concentrations a paradoxical decrease in the lysosomal ion trapping capacity of cells was observed (Figure 3.18). This effect was presumed to reflect the concomitant dose-dependent alkalization of lysosomes caused by imipramine. Interestingly, ammonium chloride

and other hydrophilic amine-containing compounds have been well documented as causing an osmotically driven expansion of the lysosomal compartment (Ohkuma and Poole 1981; Morissette, Lodge et al. 2008). To investigate whether ammonium chloride causes an increase in the lysosomal ion trapping capacity of cells, similar to imipramine, cells were treated with a range of ammonium chloride concentrations for 24 hours and in the last 2 hours LTR was added and cellular accumulation of the probe was measured and normalized to untreated control (Figure 5.1). Unlike imipramine, ammonium chloride failed to cause any appreciable increase in the lysosomal ion trapping capacity of cells at any dose tested. Rather, ammonium chloride showed only a dose-dependent decrease in lysosomal ion trapping presumed to represent the well-established alkalinizing effect of this amine on lysosomes (Ohkuma and Poole 1978). The inability of ammonium chloride to cause an increase in the lysosomal ion trapping capacity of cells, despite the well documented ability to cause an expansion of the lysosomal compartment, in the form of cytoplasmic vacuolization, suggests that the expanded compartment is not capable of participating in lysosomal ion trapping because the compartment doesn't have an intact pH gradient.

5.3.2. Lysosomal volume expansion through lysosomal lipid accumulation and cytoplasmic vacuolization

The expansion of the lysosomal compartment in cells treated with hydrophilic and hydrophobic amine-containing compounds presumably occurs through distinct molecular mechanisms. Although a thorough understanding of either mechanism is lacking, the expanded compartment that results is hypothesized to be distinctly different even though recent work has attempted to describe them as occurring through the

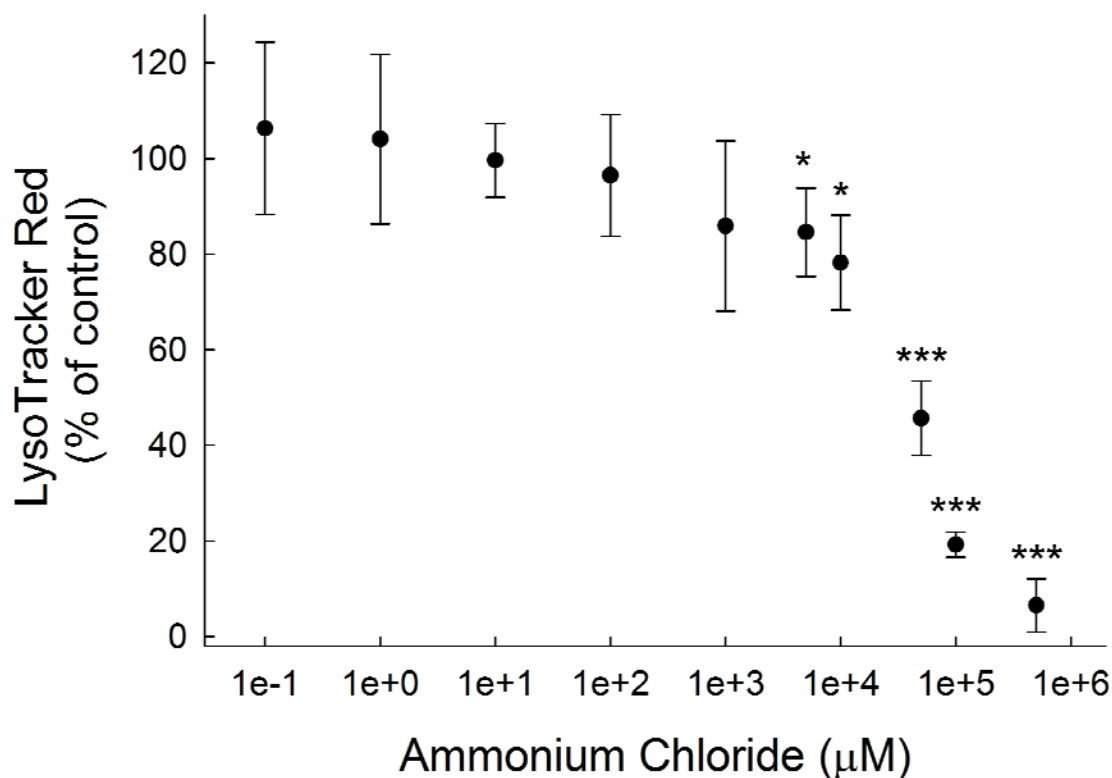


Figure 5.1. Dose-dependent decrease in lysosomal ion trapping capacity following hydrophilic amine-containing drug treatment. HeLa cells were treated for 24 hours with a range of ammonium chloride concentrations between 0 and 500 mM. In the last 2 hours, 200 nM LTR was added. Samples were subsequently lysed and measured for cell associate fluorescence. Cellular accumulation of LTR was normalized to percent vehicle-treated control. Ammonium chloride caused a dose-dependent decrease in the cellular accumulation of LTR. Cellular accumulation of LTR as a percent of the vehicle treated control cells is represented as mean \pm S.D of six values for each sample (*, $p < .05$; ***, $p < .001$ by Student's t test).

same osmotically driven mechanism (Morissette, Ammoury et al. 2009). Cytoplasmic vacuolization induced by hydrophilic amines is believed to occur through an osmotic expansion of the lysosomal compartment driven by high lysosomal amine concentrations (Ohkuma and Poole 1981), whereas lysosomal lipidosis induced by hydrophobic amines is believed to occur through the inhibition of lysosomal lipid metabolism (Halliwell 1997). Cytoplasmic vacuolization is visual by light microscopy, reversible by treatment with membrane-impermeable osmotic agents and is mostly devoid of lipids in the vacuole lumen (Morissette, Lodge et al. 2008). On the other hand, the lysosomal lipidosis caused by hydrophobic amine-containing compounds results in an expanded lysosomal compartment that is not readily visible by light microscopy, but can be visualized by electron microscopy or fluorescent labeling of the lipid-laden vesicles (Brown, Sullivan et al. 1992; Fujimura, Dekura et al. 2007). These compartments are described as containing vesicular and lamellar structures representing the accumulation of undigested lipids. Therefore, the mechanism of lysosomal volume expansion and the character of the expanded compartment in drug treated cells are believed to be distinctly different for imipramine and ammonium chloride.

Using a light microscope, equipped with a high resolution differential interference contrasting (DIC) objective, cytoplasmic vacuolization following treatment with the model vacuolizing amine, 2.5 mM procainamide, was readily visible (Figure 5.2). The extremely large vacuolar structures that appear to consume the entire cytoplasm of the cell are typical. Treatment with the membrane-impermeable osmotically active sugar, mannitol, at concentrations between 200 mM and 800 mM dose-dependently inhibited

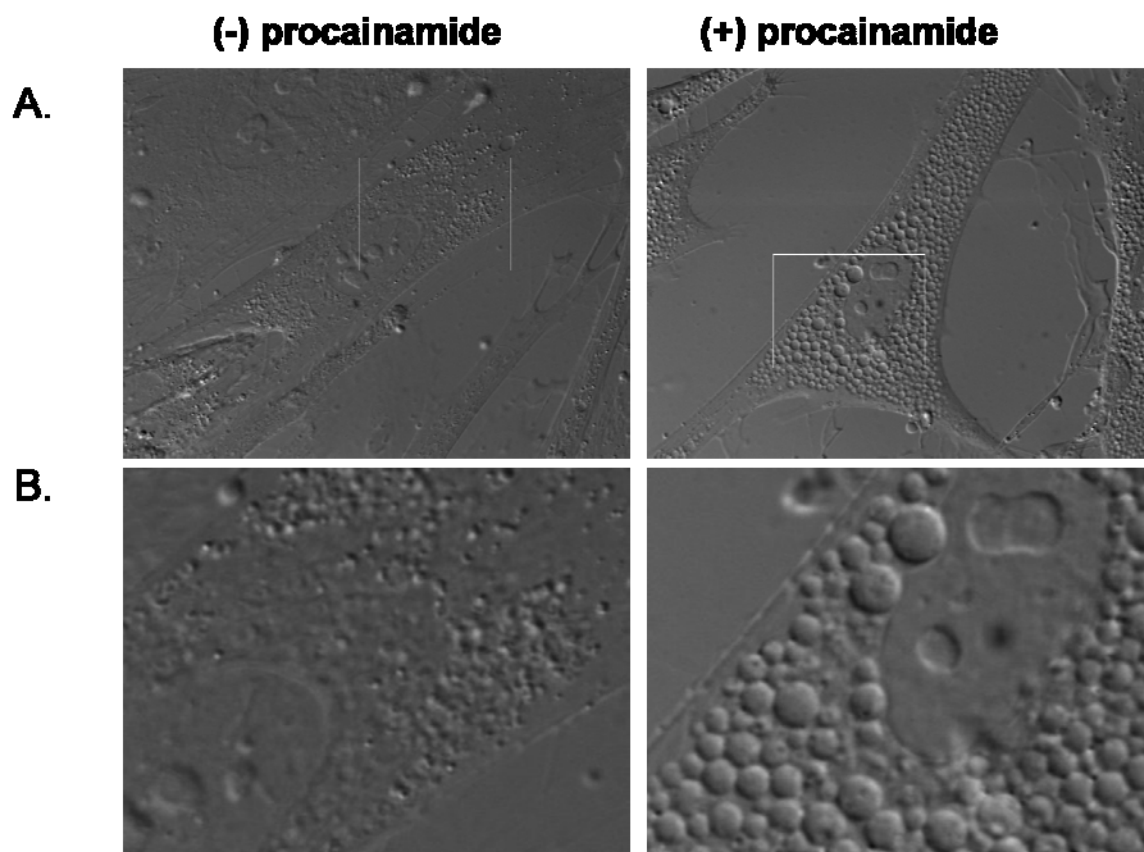


Figure 5.2. Cytoplasmic vacuolization following hydrophilic amine-containing drug exposure. Fibroblasts treated with (+), or without (-), 2.5 mM procainamide for 24 hours were imaged by DIC microscopy. (A) Cells treated with procainamide contained large perinuclear vacuolar structures that filled the cytoplasm. (B) Expansion of the highlighted insets from (A) clearly illustrates the distinct vacuolar structures that are indicative of the drug-induced cytoplasmic vacuolization.

the appearance of cytoplasmic vacuoles (Figure 5.3). Cellular staining with the lipid-dye, Nile red, failed to significantly label the interior of the procainamide-induced vacuoles (Figure 5.4). Treatment with the lipidosi-inducing hydrophobic amine, 100 μ M propranolol, resulted in no marked cytoplasmic vacuolization and the only vesicular structures present were labeled with Nile red. Therefore, in accordance with previous findings, the drug-induced cytoplasmic vacuoles are reversible by treatment with membrane-impermeable osmotically active agents and fail to show a significant level of lipid-staining within their lumen (Morissette, Lodge et al. 2008). The lack of a cytoplasmic vacuolization that is negative for Nile red staining in the lipidosi-inducing propranolol treatment supports the hypothesis that the lysosomal lipidosi is in fact distinct from the osmotically driven cytoplasmic vacuolization.

Because cytoplasmic vacuolization is a dose-dependent effect (Ohkuma and Poole 1981), a possible dose-dependent formation of cytoplasmic vacuoles following treatment with ammonium chloride (Figure 5.5) and imipramine (Figure 5.6) was evaluated. Treatment with titrated concentrations of ammonium chloride revealed a dose-dependent formation of large cytoplasmic vacuoles. Vacuolization appeared to begin at concentrations of 10 to 20 mM, but were not completely evident until a concentration of 50 mM was reached. In contrast, imipramine failed to cause a pronounced cytoplasmic vacuolization at any of the tested doses and actually significantly affected cell attachment and apparent viability at 200 μ M. A cell viability assay measuring mitochondrial dehydrogenase activity using the tetrazolium salt WST-1 confirmed that imipramine had an IC_{50} of 72 ± 3 μ M (Figure 5.7). Therefore, the hydrophobic amine, imipramine, failed to cause the cytoplasmic vacuolization induced

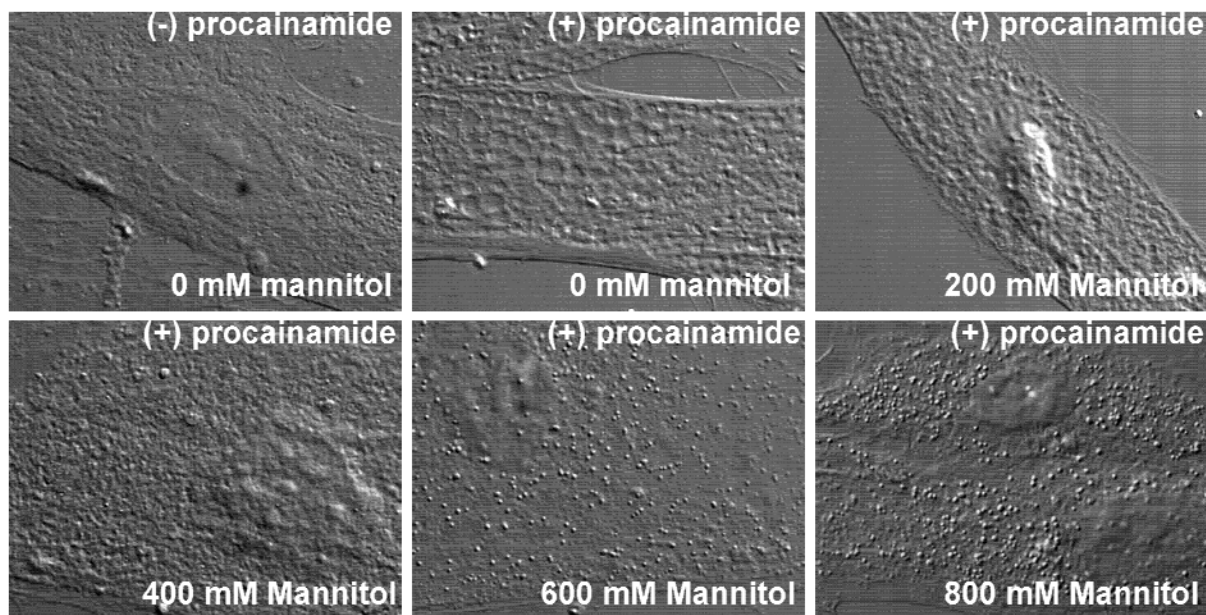


Figure 5.3. Membrane-impermeable osmotic agents reverse the drug-induced cytoplasmic vacuolization. Fibroblasts were treated for 2 hours with (+) or without (-) procainamide in the presence of mannitol at concentrations between 0 mM and 800 mM. Cells were subsequently fixed in 4% PFA and imaged by DIC microscopy. The membrane-impermeable osmotically active sugar, mannitol, inhibited the procainamide-induced cytoplasmic vacuolization in a dose-dependent manner.

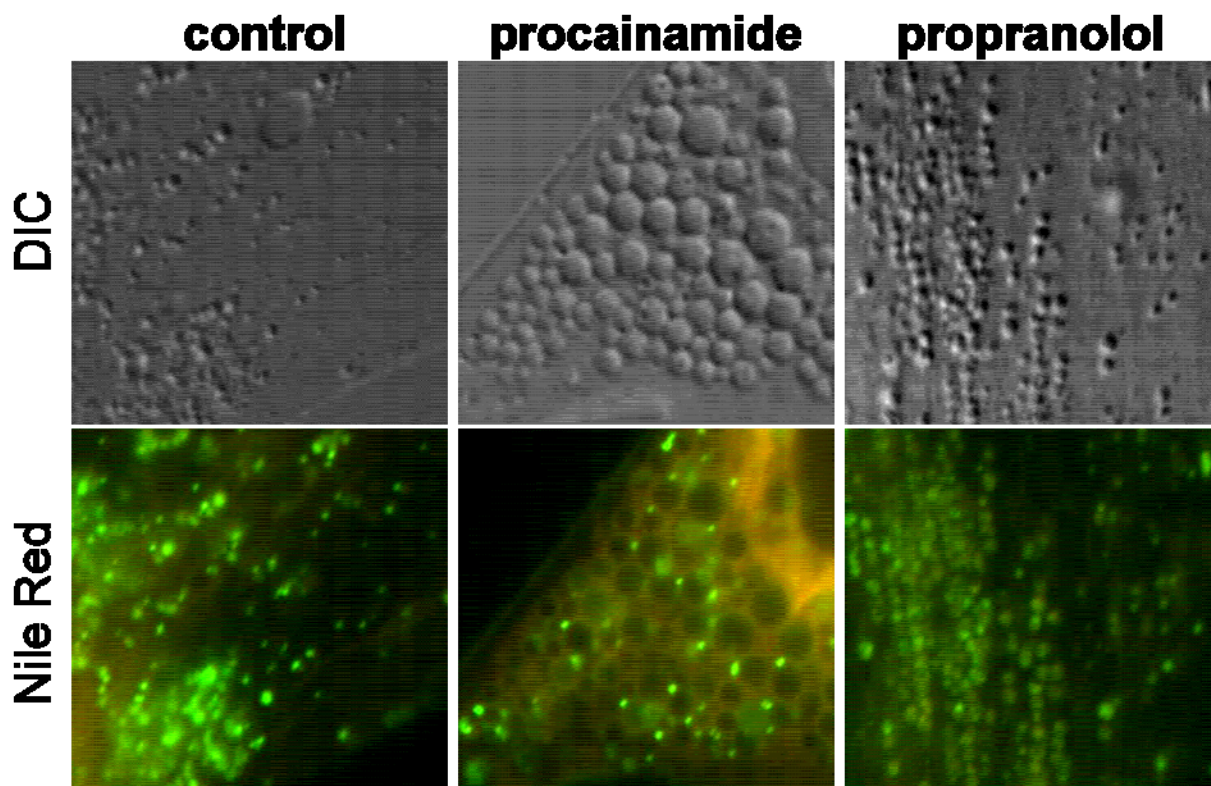


Figure 5.4. Cytoplasmic vacuoles are mostly devoid of lipids. Fibroblasts were left untreated (control) or treated with 2.5 mM procainamide or 100 μ M propranolol for 24 hours. Cells were stained with 2 μ g/ml Nile red for 30 minutes and imaged by DIC and fluorescence microscopy. Fluorescence in the green channel is indicative of a neutral lipid rich environment, whereas signal in the red channel indicates association of the dye with polar lipids. Procainamide treated cells are marked by the accumulation of large cytoplasmic vacuoles that are mostly devoid of the lipid stain. The lipidosis-inducing hydrophobic amine-drug, propranolol, failed to cause the formation of cytoplasmic vacuoles that negatively stain with the lipid dye.

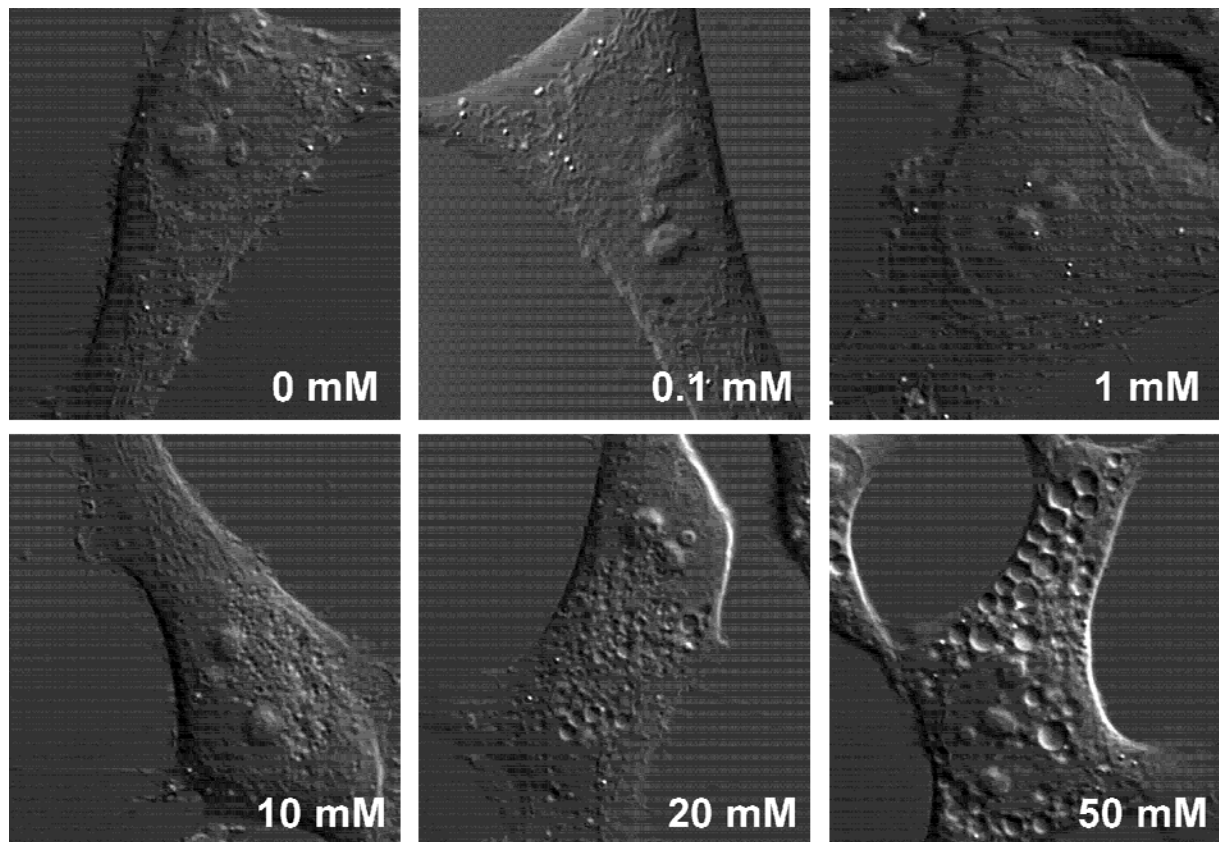


Figure 5.5. Ammonium chloride induces a dose-dependent cytoplasmic vacuolization. HeLa cells treated for 2 hours with ammonium chloride concentrations between 0 mM and 50 mM were imaged by DIC microscopy. Cytoplasmic vacuolization appeared to begin around 10 mM, but was pronounced at the 50 mM dose.

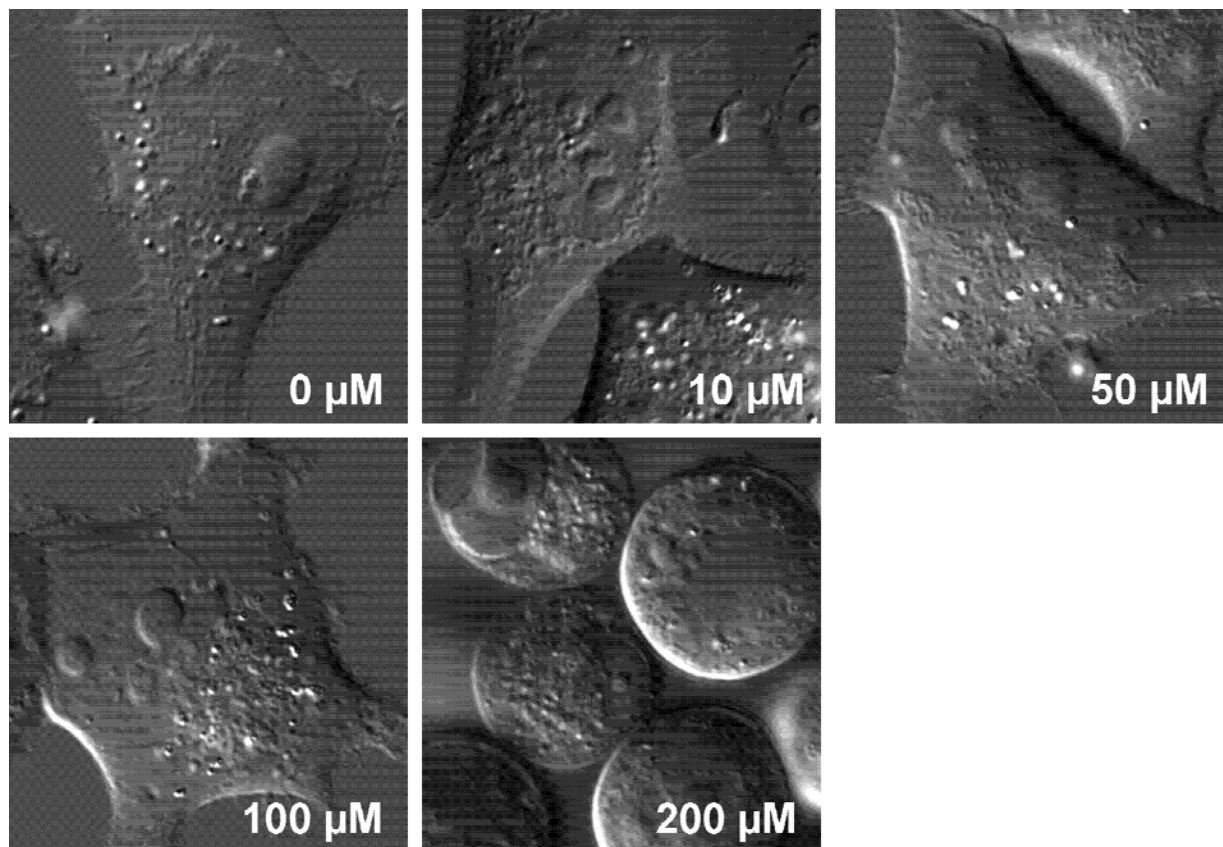


Figure 5.6. Imipramine fails to induce a cytoplasmic vacuolization at any dose tested. HeLa cells treated for 2 hours with imipramine concentrations between 0 μM and 200 μM were imaged by DIC microscopy. Cytoplasmic vacuolization was not observed at any of the tested doses. At the 200 μM dose, cells became detached from the glass surface.

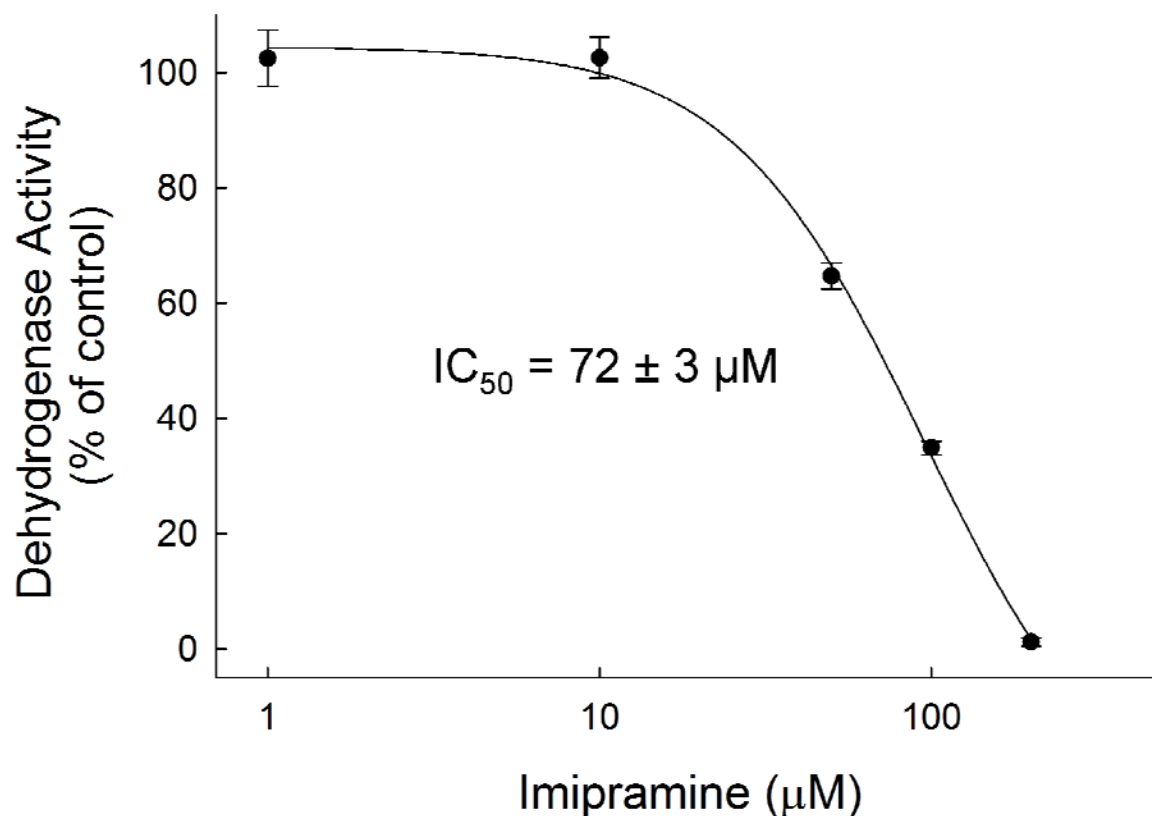


Figure 5.7. Imipramine causes a dose-dependent decrease in cell viability. HeLa cells in a 96-well plate were treated with concentrations of imipramine between 0.1 μM and 1000 μM for 24 hours. Cell viability was measured using the water-soluble tetrazolium salt (WST-1) to measure mitochondrial dehydrogenase activity. Percent dehydrogenase activity normalized to untreated cells is plotted as a function of the imipramine treatment concentration (log scale). The data was fit with a 4-parameter Hill equation using SigmaPlot from which the concentration of imipramine resulting in 50% inhibition of dehydrogenase activity (IC_{50}) was calculated. Dehydrogenase activity as a percent of the vehicle treated control cells is represented as mean \pm S.D of four values for each data point.

by ammonium chloride, even at the highest dose tested and further supports the hypothesis that the lysosomal volume expansion described as cytoplasmic vacuolization is distinct from the lysosomal lipidosis.

The failure of imipramine to cause cytoplasmic vacuolization may indicate that it doesn't stimulate the NPC1-dependent fusion of late endosomes and lysosomes as has been suggested with hydrophilic amine-containing compounds (Kaufmann and Krise 2008), like ammonium chloride. In fact, it was found that hydrophobic amines, like imipramine, actually inhibit the NPC1-dependent fusion of late endosomes and lysosomes necessary to form the amine-induced vacuoles and may actually inhibit the formation of these enlarged vacuoles. Similarly, if amines do in fact cause cytoplasmic vacuolization through an osmotically driven process then the concentration of the amine in the lysosome would be crucial in increasing the osmotic pressure within the lysosome (Ohkuma and Poole 1981). Therefore, imipramine may simply fail to reach the intralysosomal concentrations necessary to cause cytoplasmic vacuolization before it becomes cytotoxic. Regardless of the mechanism, the hydrophobic amine failed to cause the cytoplasmic vacuolization caused by high millimolar concentrations of the hydrophilic amine, suggesting that hydrophobic amines don't cause lysosomal volume expansion through the same mechanism as hydrophilic amines.

The lysosomal accumulation of lipids which is typical of the drug-induced lipidosis can be assessed by measuring the cellular accumulation of the fluorescently-labeled phospholipid analog, NBD-PE (Tomizawa, Sugano et al. 2006). Accumulation of the lipid-analog was measured in cells grown for 96 hours in the presence of 50 μ M NBD-PE that were treated with vehicle alone (control), 10 μ M imipramine or 10 mM

ammonium chloride (Figure 5.8). Although both treatments significantly increased cellular accumulation of NBD-PE, imipramine treatment resulted in a far greater (i.e., 285%) increase as compared to ammonium chloride (i.e., 42%). The mild lipidoses caused by ammonium chloride may result from the alkalinization of lysosomes at the high treatment concentration used. Lysosomal alkalinization has been shown to decrease lysosomal protease activity (Ohkuma, Chudzik et al. 1986) and may similarly decrease lysosomal lipase activity, since these enzymes have pH dependent activities with an acidic optimum (Sando and Rosenbaum 1985). The inability of the hydrophilic amine to cause the severe lysosomal lipidoses seen with imipramine isn't surprising based on numerous structure activity relationships that have shown hydrophobicity to be a key predictor for the drug-induced lysosomal lipidoses (Tomizawa, Sugano et al. 2006; Natalie, Margino et al. 2009). The failure of these studies to see a lipidoses with hydrophilic amines may have been related to the lower drug doses they restricted their studies to as compared to the high dose used here. Together the importance of hydrophobicity in causing the lipidoses suggests that the hydrophobic portion of the molecule plays a key role in its ability to cause lysosomal volume expansion through inhibition of lipid metabolism. Whether this is through facilitating interactions of the drug with intralysosomal targets, such as phospholipids, the luminal membrane or specific proteins would require further mechanism-based studies.

The distinction between the lysosomal volume expansion by lysosomal lipid accumulation and cytoplasmic vacuolization supports the hypothesis that these cytopathologies occur through distinct molecular mechanisms and may be related to the relative hydrophobicity of the amine-containing compound. Therefore, the failure of

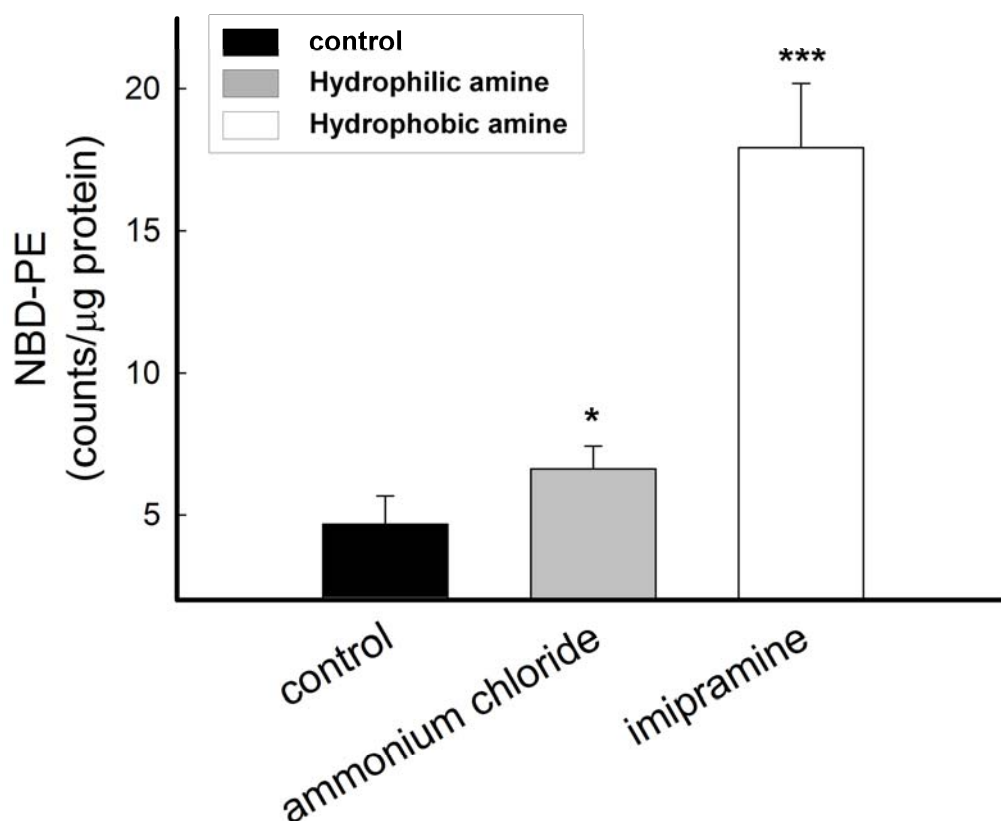


Figure 5.8. Amine-containing drug treatment results in increased cellular accumulation of the phospholipid analog, NBD-PE. HeLa cells were left untreated (control) or treated with 10 mM ammonium chloride or 10 μ M imipramine in growth media containing 50 μ M of the fluorescent phospholipid analog, NBD-PE. After 96 hours cells were lysed and cell associated NBD-PE was measured by fluorescence and normalized to cellular protein content. Although imipramine caused the greatest increase in NBD-PE accumulation, both drug treatments resulted in a statistically significant increase in probe accumulation. Cellular accumulation of NBD-PE in RFU per μ g of protein is represented as mean \pm S.D of at least three values for each sample (*, $p < .05$; ***, $p < .001$ by Student's t test).

ammonium chloride to cause the increased lysosomal ion trapping capacity of cells may reflect its inability, or decreased potency, in causing a significant expansion of the lysosomal compartment through an increase in the lysosomal accumulation of lipids. The importance of a lysosomal expansion via the lipidosis may reflect a lysosomal volume expansion that can occur at doses inferior to those necessary to cause alkalization of lysosomes, while cytoplasmic vacuolization may only occur at doses far above those necessary to cause a marked alkalization of lysosomes.

5.3.3. Lysosomal alkalization following treatment with hydrophobic and hydrophilic amine-containing compounds

In addition to expansion of the lysosomal compartment the other effect of amine-containing drugs on lysosomes that can significantly affect the lysosomal ion trapping capacity of cells is alkalization of lysosomes. Therefore, the combination of the lysosomal volume expanding and lysosomal alkalizing effects of these drugs is presumed to dictate their impact on the lysosomal ion trapping capacity of cells. If ammonium chloride and other hydrophilic amine-containing compounds cause lysosomal alkalization at doses well below those necessary to cause expansion of the lysosomal compartment, then, an increase in the lysosomal ion trapping capacity would not be expected to occur. Similarly, the increased potency of hydrophobic amine-containing compounds to cause the lysosomal volume expansion at concentrations below those necessary to cause lysosomal alkalization may explain why the increase in lysosomal ion trapping capacity seen at lower concentrations of imipramine is reversed with increasing concentrations (see Figure 3.18).

Alkalinization of lysosomes resulting from exposure to amine-containing compounds was assessed using an indirect assay that relies on short-term exposure to amine-containing drugs along with the fluorescent substrate for lysosomal ion trapping, neutral red. This assay relies on the simple assumption that the cellular uptake of the lysosomotropic amine dye, neutral red, is directly dependent on the presence of an intact lysosomal pH gradient. Therefore, alkalinization of lysosomes will result in a decrease in the cellular uptake of neutral red. The short-term drug treatments were used to prevent the possible confounding effects of drug-induced expansion of the lysosomal compartment, which has been shown to be minimal with short exposure times, as illustrated in Chapter 3. The cellular uptake of neutral red results in pronounced vesicular staining and cellular accumulation that is prevented by co-treatment with the V-ATPase inhibitor concanamycin A (Figure 5.9). These results confirm the importance of lysosomal pH in neutral red uptake into cells as well as the uptake into vesicular structures that are presumed to be lysosomes. The assay was designed for a 96-well plate format in which cells are incubated with different drugs at a range of concentrations for one hour followed by the addition of neutral red with a one-hour incubation under normal growth conditions. After washing and lysing the cells, the cell-associated neutral red in each sample is quantified by fluorescence and the percentage inhibition in cellular uptake is plotted against the drug concentration (Figure 5.10). The dibasic amine-containing drug chloroquine is well known for its ability to cause lysosomal alkalinization in the low micromolar range (Poole and Ohkuma 1981) and shows a sigmoidal dose-response in the inhibition of neutral red uptake. The resulting data were fitted using the 4-parameter Hill equation and the concentration

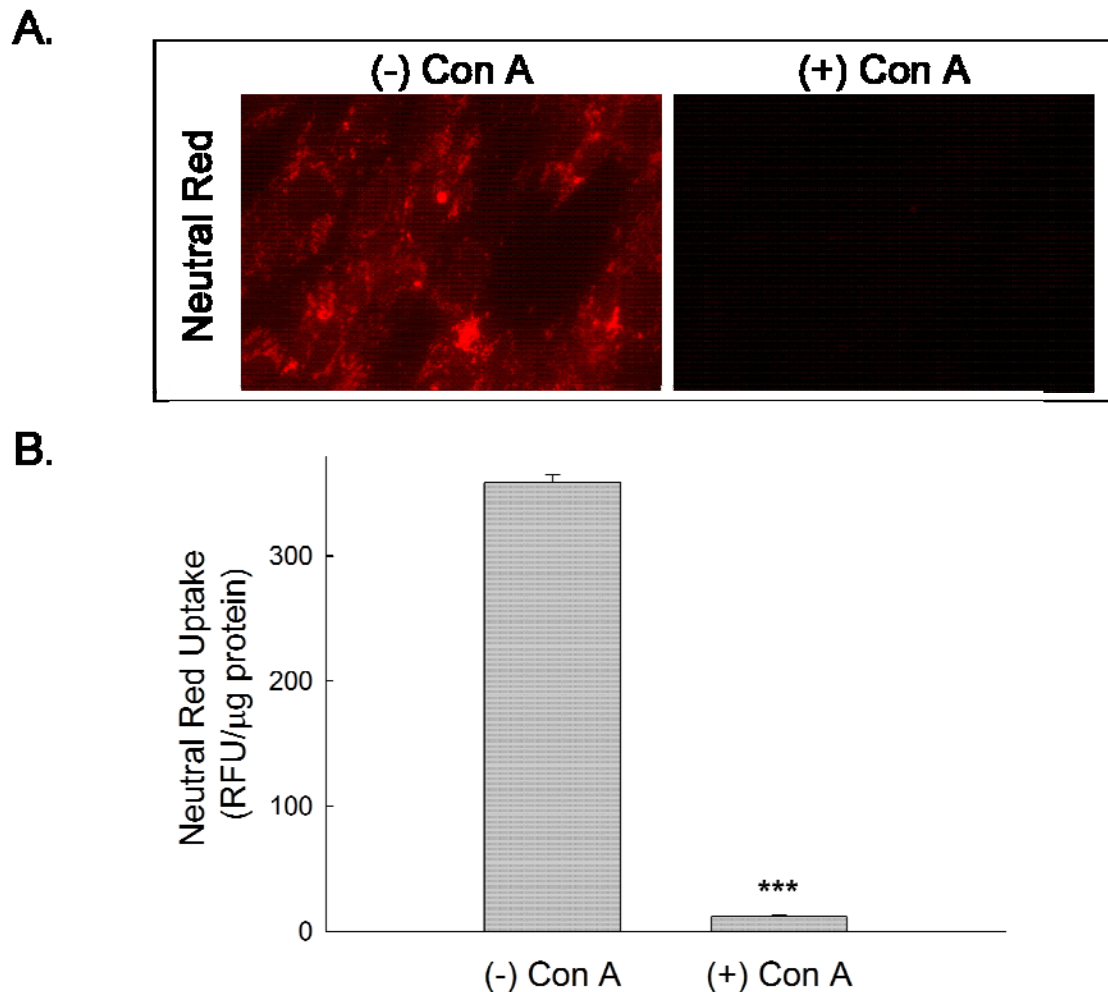


Figure 5.9. Neutral red accumulates in cells by ion trapping in lysosomes. Fibroblasts were treated with neutral red followed by treatment with (+) or without (-) the V-ATPase inhibitor concanamycin A (Con A). (A) Fluorescence micrographs show a punctate vesicular staining that is inhibited by treatment with Con A. (B) Total cellular accumulation of the fluorescent molecule was also inhibited by Con A treatment. Together this data supports neutral red accumulation in lysosomes by ion trapping that accounts for about 97% of the total cellular accumulation of the fluorophore. Cellular uptake of neutral red in RFU per μg of protein is represented as mean \pm S.D of three values for each sample (***, $p < .001$ by Student's t test).

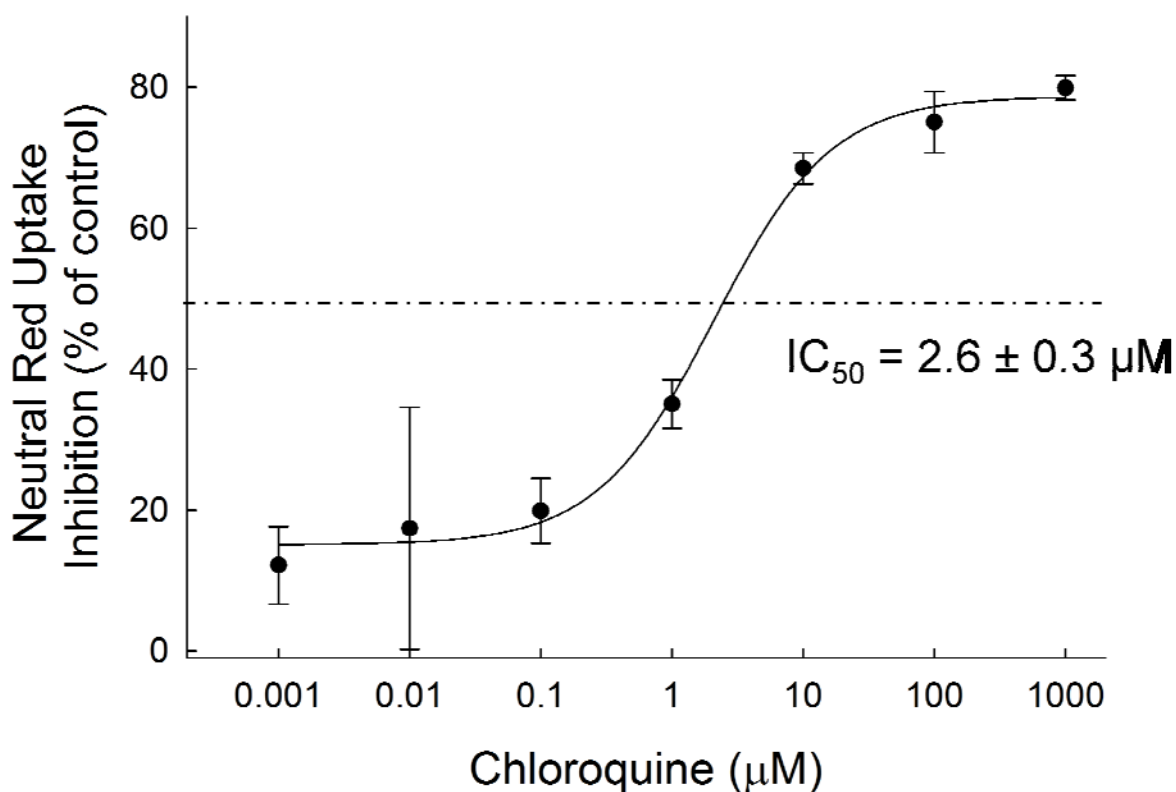


Figure 5.10. Dose-dependent inhibition of cellular uptake of neutral red by the lysosomotropic amine-containing drug, chloroquine. Fibroblasts were treated with titrated concentrations of chloroquine in triplicate for a total of 2 hours. After the first hour, 10 μ M neutral red was added and allowed 1 hour for uptake. Cells were subsequently washed and cell-associated fluorescence was measured. Percent inhibition of neutral red uptake was plotted against chloroquine dose (log scale). The data were fitted with a 4-parameter Hill equation. The resulting equation was used to calculate the concentration of chloroquine necessary to inhibit total neutral red uptake by 50% (IC_{50}). The IC_{50} value represents the potency of the drug in inhibiting neutral red uptake presumably through alkalinization of lysosomes. Inhibition of cellular uptake of neutral red as a percent of the vehicle-treated control cells is represented as mean \pm S.D of three values for each sample.

necessary to cause 50% inhibition of neutral red uptake was calculated (i.e., IC_{50}) using the resulting equation. The IC_{50} value for chloroquine was found to be $2.6 \pm 0.3 \mu M$ in agreement with the high potency lysosomal alkalinizing effect of this drug.

Next, several amine-containing compounds that are either confirmed or suspected substrates for lysosomal ion trapping with pK_a values near neutrality were chosen to be representative of amine-containing drugs that can be further characterized as hydrophobic ($\log P > 2.5$) or hydrophilic ($\log P < 2.5$) (Figure 5.11). Similar to chloroquine, these drugs were measured for their dose-dependent inhibition of neutral red uptake and the resulting data were fitted to the Hill equation from which IC_{50} values were calculated. The IC_{50} values represent an indirect measurement of lysosomal alkalinization, with decreased IC_{50} values indicating increased potency in causing lysosomal alkalinization and vice versa. The compounds are listed in Table 5.1 along with their respective pK_a , $\log P$ and IC_{50} values. A plot of the \log_{10} transformed IC_{50} versus the $\log P$ of each compound is presented (Figure 5.12). A Pearson product-moment correlation was calculated and a significant correlation between the potency of the compound in causing lysosomal alkalinization (i.e., $\log IC_{50}$) and its hydrophobicity (i.e., $\log P$) was observed ($r = -0.82$, $n = 10$, $p < 0.01$).

These results confirm that hydrophobic amine-containing drugs are far more potent at causing lysosomal alkalinization with IC_{50} values in the low micromolar range, as compared to the high micromolar to low millimolar range seen with the hydrophilic compounds. Although the hydrophobic compounds are much more potent at causing lysosomal alkalinization, they also appear to cause expansion of the lysosomal compartment in the low micromolar range that allows for the increased lysosomal ion

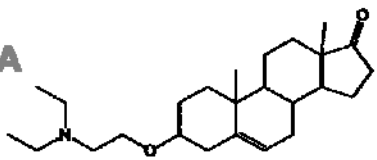
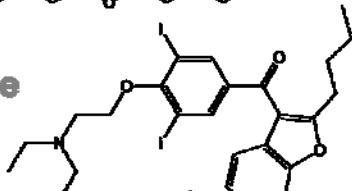

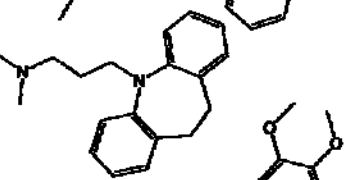
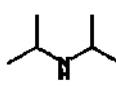
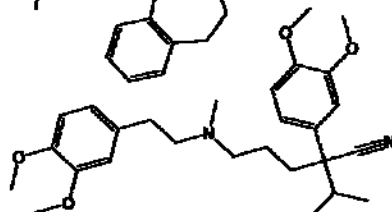

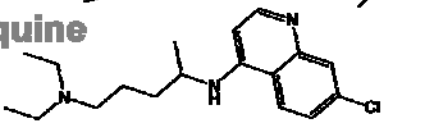

Hydrophobic	Hydrophilic
U18666A 	ammonia <chem>N</chem>
amiodarone 	isopropylamine 
imipramine 	diisopropylamine 
verapamil 	imidazole 
chloroquine 	morpholine 

Figure 5.11. Lysosomotropic amine-containing drugs are divided into two groups based on their relative hydrophobicity. Hydrophobicity is predicted by $\log P$ values determined using the $\log P$ plug-in from the MARVIN software freely available at www.chemaxon.com. Compounds with $\log P < 2.5$ are considered hydrophilic and compounds with $\log P > 2.5$ are termed hydrophobic.

Compound	logP	pKa	IC₅₀ ± SD (μM)
Morpholine	-0.36	8.51	800 ± 600
Imidazole	-0.12	6.97	1500 ± 500
Ammonia	-0.98	8.86	1600 ± 300
Isopropyl amine	0.16	10.43	1080 ± 22
Diisopropyl amine	1.39	10.72	3000 ± 3800
U18666A	4.73	9.41	14 ± 2
Imipramine	4.34	9.20	9 ± 7
Verapamil	5.11	9.68	60 ± 40
Amiodarone	7.76	8.47	15 ± 6
Chloroquine	3.93	7.29,10.32	2.6 ± 0.3

Table 5.1. Lysosomotropic amine-containing compounds assessed for causing lysosomal alkalinization using the neutral red uptake inhibition assay. Each compound is listed along with its predicted logP and pKa values determined using plug-ins from the MARVIN software freely available at www.chemaxon.com. The IC₅₀ is the concentration of compound needed to inhibit the cellular uptake of neutral red by 50%. Dose-dependent inhibition curves were generated experimentally and fitted with the Hill equation in SigmaPlot. The absolute IC₅₀ was calculated using the resulting equation and represented as the mean ± S.D of three values for each compound.

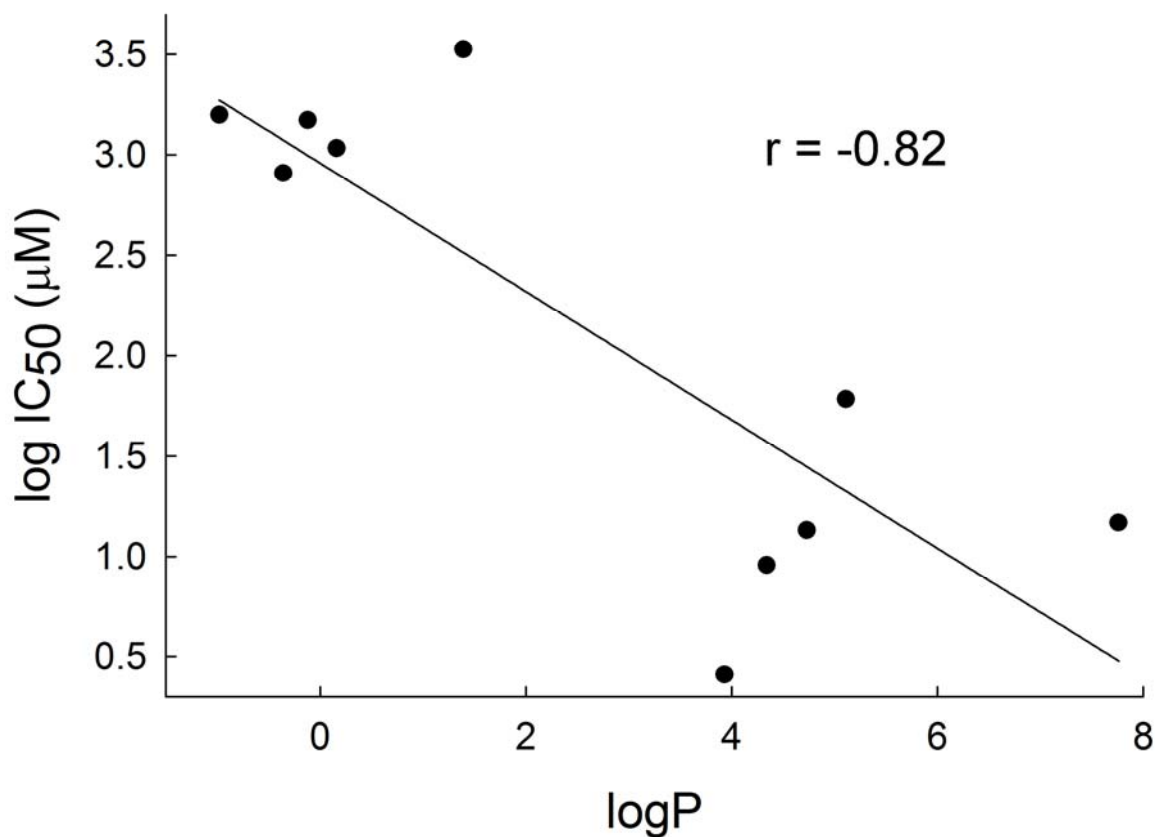


Figure 5.12. Amine-drug hydrophobicity correlates with potency in causing alkalinization of lysosomes. A plot of the IC_{50} values (log scale) and $\log P$ values from Table 2.1 illustrates the relationship between hydrophobicity ($\log P$) and potency in causing lysosomal alkalinization (IC_{50}). A Pearson's correlation of the log of the IC_{50} versus $\log P$ was conducted using Wessa statistics software ($r = -0.82$, $n = 10$, $p < 0.01$), freely available online at www.wessa.net.

trapping capacity observed with these compounds. However the hydrophilic amine-containing compounds cause lysosomal alkalization at concentrations well below the middle to high millimolar concentrations necessary to cause cytoplasmic vacuolization and suggests that despite the ability of these compounds to cause an expansion of the lysosomal compartment they are much more potent at causing alkalization of lysosomes and therefore fail to cause the increase in lysosomal ion trapping capacity of cells seen with the hydrophobic amines.

In contrast to the increased lysosomal ion trapping capacity observed following treatment with imipramine, ammonium chloride failed to cause a significant increase in the lysosomal ion trapping of cells as measured by the cellular accumulation of LTR. In chapter 3, the increase in lysosomal ion trapping capacity observed with imipramine was found to result from an expansion of the lysosomal compartment that was related to the lysosomal lipidosis caused by imipramine. Despite the ability of ammonium chloride to cause an expansion of the lysosomal compartment (i.e., cytoplasmic vacuolization), it was found to cause lysosomal alkalization at doses far inferior to those needed to cause cytoplasmic vacuolization. Therefore, the failure of ammonium chloride to cause an increase in the lysosomal ion trapping capacity of cells appears to be due to a concomitant alkalization of lysosomes.

5.4. Conclusions

In this chapter the effect of hydrophobic ($\log P > 2.5$) and hydrophilic ($\log P < 2.5$) amine-containing compounds on the structural and functional properties of lysosomes related to the lysosomal ion trapping capacity of cells is evaluated. Measurement of the

lysosomal ion trapping capacity of cells in the form of LTR accumulation following treatment with the model hydrophilic amine, ammonium chloride, failed to show an increase in the lysosomal ion trapping capacity of cells, but rather simply a dose-dependent inhibition. Lysosomal volume expansion in the form of cytoplasmic vacuolization was evaluated by DIC microscopy and appears to be distinct from the lysosomal lipidosis as determined by the absence of vacuole staining with the lipid marker, Nile red. The model hydrophilic amine, ammonium chloride, was found to cause cytoplasmic vacuolization, but requires an extremely high dose (i.e., 50 mM). In contrast, the model hydrophobic amine, imipramine, failed to induce the cytoplasmic vacuolization at doses up to 200 μ M. Imipramine was found to be dose-limited by the compounds relative cytotoxicity ($IC_{50} = 72 \mu$ M). The lysosomal lipidosis evaluated by the cellular accumulation of the fluorescent phospholipid analog, NBD-PE, revealed that high-dose ammonium chloride causes an increase in lysosomal lipid accumulation but fails to cause the intense accumulation that is seen following low micromolar doses of imipramine. Using a series of 9 lysosomotropic amine-containing compounds it is found that, in agreement with a previous report in isolated lysosomes (Ishizaki, Yokogawa et al. 2000), the hydrophobic compounds are significantly more potent inducers of lysosomal alkalinization as compared to the hydrophilic compounds. Together these results support the hypothesis that hydrophobic and hydrophilic amine-containing compounds distinctly differ in their ability to alter the functional and structural properties of lysosomes. Hydrophobic amine-containing compounds are potent at causing lysosomal alkalinization and lysosomal lipidosis, but fail to cause cytoplasmic vacuolization. Hydrophilic amine-containing compounds are weak inducers of

lysosomal alkalization and lysosomal lipid accumulation, but capable of causing cytoplasmic vacuolization at high doses. Despite the ability of hydrophilic amine-containing compounds to cause an apparent expansion of the aqueous volume of lysosomes (i.e., cytoplasmic vacuolization) their failure to cause an increase in the lysosomal ion trapping capacity of cells is presumed to result from the concomitant alkalization of lysosomes that negates any potential increase in lysosomal ion trapping.

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Chapter 6

Summary, conclusions and future directions

6.1. Summary and conclusions

With advanced techniques to determine the subcellular distribution of drugs it is becoming evident that drugs often fail to show a homogenous distribution within the cell, but rather tend to accumulate within specific subcellular compartments (Baik and Rosania 2011). The tendency to accumulate to a high degree within these compartments has been demonstrated to affect the pharmacokinetic and pharmacodynamic properties of the drug, but have also been observed to result in perturbations in the normal structural and functional properties of the organelle (Kaufmann and Krise 2007). This ability to affect the physiological function of the organelle has raised concerns about possible toxicological ramifications, but has also resulted in hypothesized therapeutic applications. Therefore, it is important to gain an understanding of the mechanisms through which these drugs affect the properties of the organelle, as well as the potential therapeutic implications of these effects.

The subcellular disposition of weakly basic amine-containing drugs in lysosomes has been well documented as affecting the functional and structural properties of the organelle (Lullmann-Rauch 1979; Ohkuma and Poole 1981; Poole and Ohkuma 1981). In fact, the ability of amine-containing drugs that accumulate in lysosomes to cause a dose-dependent alkalinization of the lysosome has been demonstrated to result in numerous biological effects of potential pharmacological and toxicological relevance (Baxt 1987; Christensen, Myers et al. 2002; Savarino, Boelaert et al. 2003; Talloczy, Martinez et al. 2008; Matsui, Ito et al. 2010; Ashfaq, Javed et al. 2011), including intracellular distribution-based drug-drug interactions (Daniel 2003). Amine-containing drugs are also known to cause the lysosomal accumulation of lipids, resulting in the

drug-induced syndrome known as phospholipidosis (Lullmann-Rauch 1979). The phenotypic similarity between the drug-induced lipidosis and the lysosomal lipid storage disease, Niemann-Pick type C (NPC) disease, has resulted in the wide-spread use of these drugs in inducible models of the disease (Rodriguez-Lafrasse, Rousson et al. 1990). Interestingly, cells isolated from patients suffering from NPC disease have been characterized as having an expanded aqueous volume of the lysosomal compartment (Kopitz, Harzer et al. 1996). Therefore, a similar effect was predicted to occur following the disease-like lipidosis caused by many of these amine-containing drugs.

An expansion of the aqueous volume of the lysosomal compartment would be expected to impact the cellular accumulation of drugs by ion trapping in lysosomes (de Duve, de Barsy et al. 1974) and would illustrate a novel mechanism through which drugs can interact at the subcellular level. Therefore, the influence of the drug-induced lysosomal accumulation of lipids on the cellular accumulation of weakly basic-amine containing drugs and the mechanisms involved were the focus of this thesis. In this work, the drug-induced expansion of the lysosomal compartment was evaluated for its impact on the lysosomal ion trapping capacity of cells and lysosomal enzyme activity.

Hydrophobic amine-containing drugs that are well established as causing the lysosomal accumulation of lipids (Roff, Goldin et al. 1991) were found to cause an increase in the lysosomal ion trapping capacity of cells similar to that seen in cells isolated from patients suffering from NPC disease. Using the model hydrophobic amine, imipramine, the increased lysosomal ion trapping was found to occur through a time- and temperature-dependent process consistent with an energy-dependent remodeling of the cell resulting in an expansion of the aqueous volume of the lysosomal

compartment. Reversal of the cellular lipidosis caused a reduction in the apparent volume of the lysosomal compartment and suggested that the drug-induced expansion of the lysosomal compartment is secondary to the cellular accumulation of lipids, rather than a direct effect of the drug. These findings argue that hydrophobic amine-containing drugs do not cause an expansion of the lysosomal compartment by directly inhibiting the trafficking of lysosomes as has been previously hypothesized (Goldman, Funk et al. 2009). Rather these findings would support the hypothesis that these drugs cause the lysosomal accumulation of lipids and that the change in the lipid content of lysosomes causes the observed inhibition in the vesicle-mediated efflux trafficking of lysosomes and therefore results in an expanded lysosomal compartment.

The possibility that lysosomal volume expansion, following hydrophobic amine-treatment, could result in a decrease in the cellular activity of lysosomal enzymes was also evaluated. An expansion of the lysosomal compartment was hypothesized to possibly decrease lysosomal enzyme activity by decreasing their apparent concentration within the lysosome. Although changes in lysosomal pH have been shown to impact lysosomal hydrolase activity (Ohkuma, Chudzik et al. 1986), the impact of lysosomal volume expansion was previously unknown and, if found, was hypothesized to potentially contribute to the drug-induced accumulation of undigested lipids in lysosomes. Measurements of lysosomal enzyme activity, using both exogenously supplied protein and a specific substrate of cathepsin B, revealed no change in enzymatic activity. Therefore, in contrast to lysosomal pH changes, lysosomal volume expansion does not appear to have a significant effect on lysosomal enzyme activity. The lack of an effect on lysosomal enzyme activity may reflect an

increase in enzyme expression concomitant with the expansion in the lysosomal volume.

In addition to the expansion of the lysosomal compartment that was observed here, hydrophilic amine-containing drugs that are not typically associated with the lysosomal accumulation of lipids (Tomizawa, Sugano et al. 2006), have also been illustrated as causing expanded vacuolar compartments that are of lysosomal origin and often referred to simply as cytoplasmic vacuoles (Morissette, Lodge et al. 2008). This expanded compartment was determined to be distinct from the expanded compartment resulting from the lipidosis-inducing, hydrophobic amine-drug treatment and was not found to increase the capacity of cells to accumulate secondarily applied amines by ion trapping. The model hydrophilic amine, ammonium chloride, caused the formation of cytoplasmic vacuoles, but failed to increase the lysosomal ion trapping capacity of cells. This failure to increase the lysosomal ion trapping of cells despite the vacuolization was attributed to a concomitant alkalization of the lysosomal compartment. Therefore, the hydrophobicity of the amine-containing drug may be an important parameter in predicting the ability of a drug to increase the lysosomal ion trapping capacity of cells through an expansion of the lysosomal compartment.

6.2. Future directions

Future studies that focus on the mechanistic basis and the potential therapeutic implications of the lysosomal volume expansion are necessary. Although the *in vitro* findings revealed the potential for a profound effect of lysosomal volume expansion on the cellular accumulation of amine-containing drugs, *in vivo* experiments are necessary

to verify the physiological relevance of these findings. An in vivo study in rats has shown that treatment with the hydrophobic amine-containing drug, chlorphentermine, increases the cellular accumulation of a secondarily administered hydrophobic amine-containing drug, amiodarone (Reasor 1991). Although the authors attributed the increased accumulation of amiodarone to an increase in the binding to the concomitantly accumulating phospholipids, it may be that the observed increase in drug accumulation actually occurred through an expansion of the lysosomal compartment, as hypothesized in this work. Further in vivo studies measuring the effect of the drug-induced lipidosis on the cellular accumulation of an amine-containing drug that is a substrate for ion trapping in lysosomes, but is not predicted to bind phospholipids, such as methylamine, would help elucidate whether the increased drug accumulation in vivo occurs through phospholipid binding or an expansion of the aqueous volume of the lysosome. Additionally, in vivo pharmacokinetic studies on the impact of lysosomal volume expansion would be necessary to illustrate how the pharmacokinetic parameters of the drug are altered. Intuitively, an increase in the tissue accumulation of drug would result in an increased volume of distribution that would affect the necessary loading dose to achieve target plasma concentrations and would be expected to increase the half-life, if clearance remains unaffected. Such changes in the pharmacokinetic properties of a drug may or may not have therapeutic relevance especially since most lysosomotropic amine-containing drugs already have a large volume of distribution (Yokogawa, Ishizaki et al. 2002).

Future in vitro studies are necessary to understand the nature of the expanded lysosomal compartment and the biochemical mechanism through which it is formed.

Initially, an understanding of the source of the expanded compartment is needed. Presumably, an expansion of the lysosomal compartment represents a defect in the homeostatic regulation of the trafficking of lysosomes and their contents, either through an increase in their formation or a decrease in their clearance. Because material moves in and out of lysosomes through multiple pathways, it would be important to identify the disrupted pathway that results in the lysosomal volume expansion. Previous studies have illustrated that amine-containing drugs can cause that appearance of expanded vacuolar compartments that contain autophagic and lysosomal markers (Morissette, Ammoury et al. 2009), and therefore, may signify an increase in lysosomal volume mediated by an increase in the formation of acidic autophagolysosomal vacuoles, or a decrease in their clearance. Similarly, lysosomal volume expansion could originate from an increase in the biogenesis of lysosomes, or perhaps an increase in endocytic uptake or a decrease in exocytic efflux of lysosomes and their cargoes, which has also been previously hypothesized (Neufeld, Wastney et al. 1999; Kaufmann and Krise 2008).

In summary, the lysosomal volume expansion described in this thesis is characterized as a pathway through which amine-containing drugs can modify the properties of the lysosomal system. The expanded lysosomal compartment is shown to be a site for enhanced cellular accumulation of secondarily administered amine-containing drugs and reveals a novel mechanism of intracellular distribution-based drug-drug interactions.

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Appendix

Exposure of cells to hydrogen peroxide can increase the intracellular accumulation of drugs

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Exposure of Cells to Hydrogen Peroxide Can Increase the Intracellular Accumulation of Drugs

Ryan S. Funk and Jeffrey P. Krise*

Department of Pharmaceutical Chemistry, The University of Kansas,
Lawrence, Kansas 66047

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Abstract: One of the fastest growing areas of scientific research involves aspects of oxidative stress, either causes of or results from. Despite the enormous quantity of literature on the topic, surprisingly, the effects of oxidative stress on the pharmacokinetics of drugs have not been previously investigated. This is an extremely important concern, considering that the degree of oxidative stress that the human body experiences is known to be widely variable. Oxidative stress may be transiently increased, as is the case with some inflammatory episodes, or it may be chronically elevated, as is the case in some disease states, in aging, or with smokers. This report examines the influence of oxidative stress on the pharmacokinetics of model drugs utilizing cells in culture. Specifically, the effect of subtoxic, short-term exposure to hydrogen peroxide was investigated. Low micromolar, single doses of hydrogen peroxide were shown to cause dramatic increases in the apparent intracellular accumulation of model compounds with different physicochemical properties in different cell types. To examine the mechanistic basis for this, we evaluated possible hydrogen peroxide induced changes in cells including (1) intracellular pH, (2) membrane integrity, and (3) membrane fluidity (i.e., lateral membrane diffusion). We found no significant changes in pH or membrane integrity, but results were consistent with changes in hydrogen peroxide mediated reductions in lateral membrane diffusion, which we postulate facilitated the accumulation of the test substrates. Although studies presented here were all done in cell culture systems, we believe the findings could have substantial therapeutic relevance and warrant further investigations, which may provide reasons why drugs often have anomalous pharmacokinetic behavior and disproportionate dose–response relationships in certain patient populations.

Keywords: Hydrogen peroxide; drug permeability; membrane fluidity; daunorubicin; Oregon Green

Introduction

Understanding the reasons for pharmacokinetic variability of drugs represents an important area of research in which much has been accomplished. Research has provided mechanistic insight into reasons for variability that results from age, gender, diet, disease state, and genetics to name a few.^{1–4} Despite the progress that has been made, there is still much

work to do in order to achieve the ultimate goal of individualized dosing of drugs to obtain an appropriate pharmacodynamic response. Episodes in which patients

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* To whom correspondence should be addressed. Mailing address: Department of Pharmaceutical Chemistry, The University of Kansas, 2095 Constant Ave., Lawrence, KS 66047-3729. Phone: (785) 864-2626. Fax: (785) 864-5736. E-mail: krise@ku.edu.

receive hypo- or hypertherapeutic doses are still quite commonplace, some of which are known to result from alterations in drug metabolizing enzyme expression and/or activity,⁵ altered drug absorption,⁶ and changes in drug distribution,⁷ to name a few. However, the effects of hydrogen peroxide (H_2O_2), the physiological levels of which can vary considerably, on the distribution of drugs has not been previously examined to our knowledge.

H_2O_2 is not a very reactive chemical species on its own and does not oxidize most biological membranes. The molecule's oxidative capacity typically arises from its conversion to reactive hydroxyl radicals, which can occur following exposure to UV light or by interaction with transition metal ions. Levels of H_2O_2 below 50 μM are suggested to have limited toxic effects on most cell types. Interestingly, at these low concentrations the molecule is believed to function as an inter- and intracellular signaling molecule, which may provide a physiological basis for fluctuations in its concentrations.⁸ Moreover, H_2O_2 is known to fluctuate under various disease states and environmental conditions; therefore this prompted us to examine if such fluctuations could influence the accumulation of drugs in cells. Examples of factors leading to alterations in H_2O_2 levels and oxidative stress include diseases such as diabetes mellitus, arthritis, hypertension, ischemia–reperfusion, ethnicity, age, gender, and body mass index.^{9–13} A number of

these factors have long been known to influence the pharmacokinetics of drugs and therefore the pharmacodynamic response of drugs but for reasons not related to H_2O_2 ; therefore this research has the potential to allow us to obtain a more complete mechanistic insight into the observed variability.

In this work we examined the effects of physiologically relevant levels of H_2O_2 , which was introduced to cells grown in culture. The influence of the cellular uptake of two model compounds was subsequently evaluated with or without H_2O_2 pretreatment. Dramatic increases in the intracellular accumulation of drugs were observed following H_2O_2 pretreatment. To investigate the mechanism, we evaluated three likely H_2O_2 -induced changes to cells that could foster the observed increases in drug accumulation. These were (1) alterations in intracellular pH, (2) diminished plasma membrane integrity, and (3) changes in plasma membrane fluidity. Only the latter of the three was found to change to a significant extent. Collectively, the data suggest that the accumulation of drugs in cells may increase as a consequence of H_2O_2 -mediated alterations in plasma membrane dynamics.

Experimental Section

Cell lines and Reagents. Daunorubicin was purchased from Oakwood Products Inc. (West Columbia, SC). Lucifer Yellow, 2',7'-difluorescein (Oregon Green 488), 1,1'-diocetadecyl-3,3',3'-tetramethylindocarbocyanine percholate (DiIC₁₈), and (5- and 6-) carboxyl SNARF1 AM acetate ester were purchased from Molecular Probes (Eugene, OR). WST-1 was obtained from BioVision (Mountain View, CA). Hydrogen peroxide 30% in water was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO).

HL-60 cells were kindly provided by Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes, 1 mM sodium pyruvate, 0.1% penicillin, 0.1% streptomycin and were maintained at a density of 1×10^5 to 1×10^6 cells/mL. CCD-1064SK human foreskin fibroblasts were purchased from ATCC (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 $\mu g/mL$ streptomycin. All cells were grown at 37 °C in a humidified 5% CO₂ atmosphere. All experiments with cells were conducted between passage numbers four and six.

H_2O_2 Cytotoxicity Evaluations. Cell sensitivity to H_2O_2 was determined using WST-1 according to the manufacturer's protocol. Briefly, HL-60 cells were seeded in triplicate in 96-well culture plates at 5×10^4 cells/well and exposed to a range of hydrogen peroxide concentrations for a period of 2 days. At this time 10 μL of WST-1 reagent was added to each well and the plates were returned to the incubator for an additional 2 h. The absorbance of each well was measured at 450 nm in a Thermo Electron microplate reader (Waltham, MA). IC₅₀ value experiments with the fibroblasts were identical to HL-60 cells except that the seeding density was 1×10^4 cells/well and the cells were allowed to adhere

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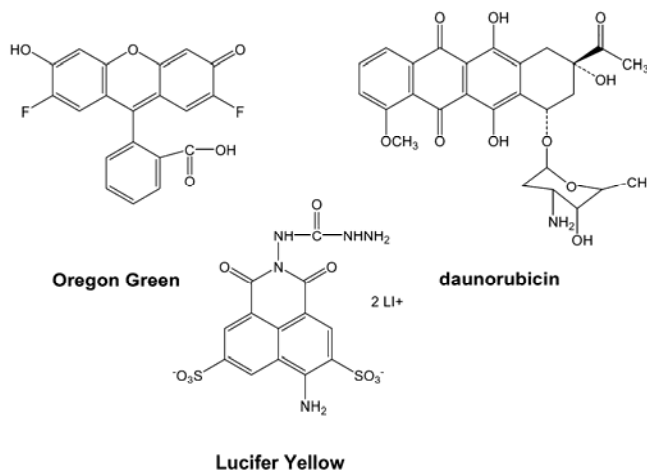


Figure 1. Structures of fluorescent molecules used to evaluate the influence of H_2O_2 on plasma membrane permeability. Both Oregon Green and daunorubicin are relatively hydrophobic and membrane permeable. Oregon Green is a weakly acidic molecule. Daunorubicin is weakly basic. Lucifer Yellow is a polar molecule known to be membrane impermeable.

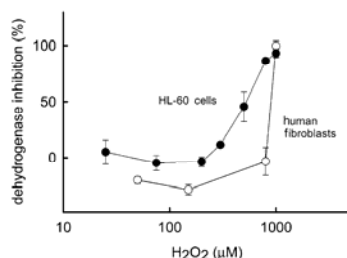


Figure 2. Cytotoxicity evaluations for H_2O_2 on the human leukemic cell line HL-60 (●) and on a human skin fibroblast (○). Concentrations of H_2O_2 used in this work were 50 μM since it is close to being physiologically relevant and did not display any significant toxicity to cells. Reported values are means \pm SD for three independent determinations.

overnight prior to hydrogen peroxide additions. IC_{50} was defined as the concentration of drug causing 50% dehydrogenase inhibition as compared to control cells.

Drug Accumulation Studies. Using results from H_2O_2 IC_{50} evaluations we chose to incubate cells with 50 μM H_2O_2 , which is a concentration that has minimal antiproliferative effects on either cell line. Fibroblasts were seeded at a density of 1×10^5 cells/mL and allowed to adhere overnight. HL-60 cells were seeded at a density of 5×10^5 cells/mL. Medium containing 50 μM H_2O_2 was added to the cells and placed in the cell culture incubator for 2 days, after which time cells were washed three times with warm PBS. Cells were subsequently exposed to Oregon Green 488 (OG, 1 μM for HL-60 cells and 2 μM for fibroblasts), daunorubicin (DNR, 50 nM, both cells), or Lucifer Yellow (LY, 2.2 mM, both cells). The cells were incubated with OG and DNR for 3 h and LY for 30 min. Following drug exposure cells were washed twice

with warm PBS to remove unincorporated drug and were viewed using a Nikon Eclipse 80i microscope equipped with epifluorescence. The FITC filter set was used to view the LY and OG. The Texas Red filter set was used to view the DNR. Images were captured using an Orca ER camera (Hamamatsu Corp., Japan) and analyzed by MetaMorph version 6.2 (Universal Imaging Corp.). With microscope settings kept constant H_2O_2 treated cells were compared to untreated control cells to observe differences in drug accumulation.

Cytosolic pH Measurements. H_2O_2 treated fibroblasts were compared to control fibroblasts to determine if the reagent caused any differences in cytosolic pH. Using (5- and 6-) carboxyl SNARF1 AM acetate ester, a pH-sensitive probe, cytosolic pH was determined using a protocol outlined in our earlier work.¹⁴ Briefly, following H_2O_2 treatment, as previously described, cells (10^7) were incubated with 4 μM (5- and 6-) carboxyl SNARF1 AM acetate ester for 1 h. Cells were then washed twice with warm PBS, resuspended in PBS (pH 7.4), and placed in a cuvette. Using a PTI spectrofluorometer, emission intensities were measured at 580 and 640 nm while using an excitation of 514 nm. The ratios of the emission intensities were used to determine the cytosolic pH as referenced.¹⁵

Fluorescence Recovery After Photobleaching (FRAP). Fibroblasts with or without H_2O_2 treatment were incubated

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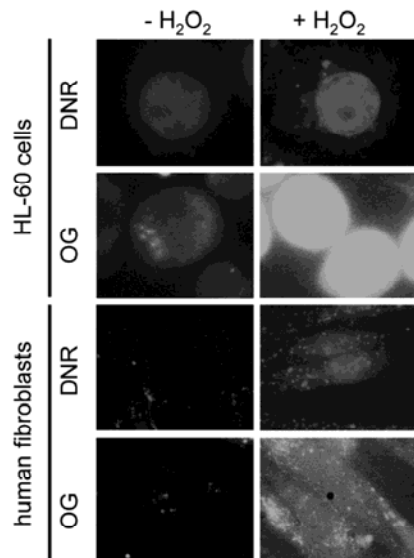


Figure 3. H_2O_2 pretreatment increases the cellular accumulation of daunorubicin (DNR) and Oregon Green (OG) in both HL-60 cells (top micrographs) and human skin fibroblasts (bottom micrographs). Designated cells ($+\text{H}_2\text{O}_2$) were incubated in culture media supplemented with $50\ \mu\text{M}$ H_2O_2 for 2 days, washed, and subsequently incubated for 3 h with Oregon Green ($1\ \mu\text{M}$ for HL-60 cells and $2\ \mu\text{M}$ for fibroblasts) or daunorubicin ($50\ \text{nM}$, both cells) in cell culture media at 37°C . Cells were subsequently washed and visualized using a fluorescence microscope. Identical microscope settings were employed to allow for meaningful comparisons. Micrographs are representative of at least 5 separate trials.

with $100\ \mu\text{M}$ DiI $_{18}$ in Hanks' Balanced Salt Solution (HBSS) for 10 min to allow the lipophilic dye to incorporate into the plasma membrane. Cells were then washed twice with warm HBSS and viewed with a LSM 510 Zeiss confocal microscope at room temperature. A defined rectangular region of interest (ROI) was set and kept constant in size throughout the experiment. A designated area of the cell plasma membrane was photobleached using a 488 nm argon laser exciting at full power for 3 s. Images were collected at 565 nm before and after the photobleaching at 100 ms intervals for a total of 11 s. Images were monitored for diffusion of unbleached dye from areas adjacent to the bleached region using ImageJ (NIH software). The fluorescence intensity of the photobleached region was quantified as a function of time using cells with and without H_2O_2 pretreatment.

Results and Discussion

Drug Selection and Evaluation of H_2O_2 Toxicity. To examine the effects of H_2O_2 on drug accumulation, two cell lines were chosen for investigation. The first was the human

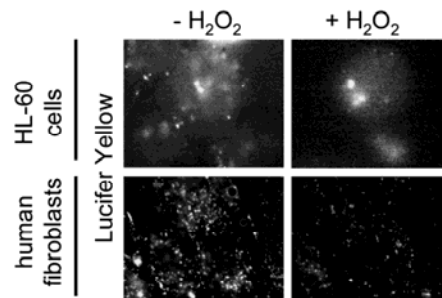


Figure 4. The influence of H_2O_2 on the plasma membrane permeability of a membrane impermeant probe Lucifer Yellow in HL-60 cells (top panels) and in human fibroblasts (bottom panels). See Figure 3 for experimental details. Lucifer Yellow was incubated with all cells at a concentration of $2.2\ \text{mM}$ for 30 min in cells with ($+\text{H}_2\text{O}_2$) or without ($-\text{H}_2\text{O}_2$) pretreatment. Intracellular punctate staining observed in the micrographs is typical of endocytic uptake of polar molecules such as Lucifer Yellow.

leukemic cell line HL-60, and the second was a normal human skin fibroblast. For each of the cell lines it was important that concentrations of H_2O_2 presented to cells were reasonably physiologic and were not significantly toxic. Others have suggested that concentrations of H_2O_2 below $50\ \mu\text{M}$ were nontoxic to most cells.⁸ To investigate the toxicity of H_2O_2 in the cells used in this study we evaluated H_2O_2 IC_{50} values. As is shown in Figure 2, the IC_{50} values for the HL-60 cells and for the human fibroblasts were well above the $50\ \mu\text{M}$ concentration used in our evaluations. Moreover, using a Trypan Blue exclusion method we show that there is negligible growth inhibition at this concentration (See Supplemental Figure 1 in the Supporting Information).

We chose two model compounds for this investigation, namely, the fluorescent weakly basic anticancer agent daunorubicin and the fluorescent weakly acidic dye Oregon Green (see Figure 1 for structures). Evaluating the fluorescence of cells with and without H_2O_2 treatment allowed us to visually document changes in drug accumulation and intracellular distribution. All settings on the fluorescence microscope were kept constant to allow for meaningful comparisons. In Figure 3, one can observe significant increases in accumulation for both compounds following H_2O_2 pretreatment. Drug accumulation was also investigated in cells experiencing hydrogen peroxide pretreatment at variable hydrogen peroxide concentrations and exposure times (Supplemental Figure 2 in the Supporting Information). There was found to be no correlation of drug accumulation with either hydrogen peroxide exposure time or hydrogen peroxide concentration.

Investigating the Mechanism for H_2O_2 -Induced Drug Accumulation. One reason for the observed increase in accumulation seen with daunorubicin could be rationalized if the cytosolic pH decreased after H_2O_2 pretreatment. This was an important consideration since hydrogen peroxide at

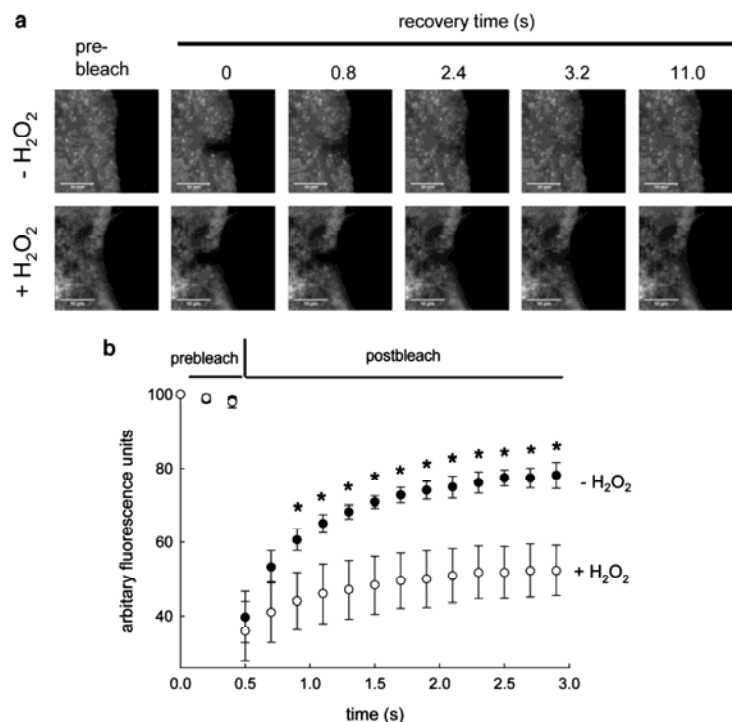


Figure 5. Analysis of membrane fluidity in fibroblasts with (+H₂O₂) or without (-H₂O₂) pretreatment by using a fluorescence recovery after photobleaching method (FRAP, see Experimental Section). Cells were treated with or without H₂O₂ as indicated on the legend. Cells were subsequently labeled with 100 μ M DiIC₁₈ for 10 min, which specifically labels the plasma membrane. (a) Micrographs of plasma membrane containing DiIC₁₈ before photobleaching (left micrographs) and micrographs of the bleached area as a function of time. The presented image is representative of at least three trials. (b) Quantitative analysis of the fluorescence intensity of the photobleached area of the plasma membrane relative to prebleached levels as a function of time are plotted. Recovery of fluorescence with control (●) and H₂O₂ pretreated cells (○) over a 3 s time period are presented. The recovery time is significantly faster in cells without H₂O₂ pretreatment (-H₂O₂) than in cells pretreated with the agent (+H₂O₂) suggesting that H₂O₂ pretreatment causes a decrease in membrane fluidity. Data are the mean of three independent experiments \pm SD. Data points for treated and control cells at H₂O₂ concentrations indicated with "*" are statistically different ($p < 0.05$).

slightly higher levels (100 μ M) had been previously shown to decrease the cytosolic pH significantly.¹⁶ If this were the case, the lower pH could make pH partitioning into the cytosol more favorable for a weakly basic drug like daunorubicin because of the increase in ionization (and a reduction in membrane permeability) when the drug enters the cytosol. One reason for selecting Oregon Green for evaluation in this study was that it was fluorescent and weakly acidic. Correspondingly, if cytosolic pH were to decrease, this could actually lead to decreases in cytosolic concentrations for this compound after H₂O₂ pretreatment, as would be in agreement with pH-partitioning theory. As is shown in Figure 3, the

levels of Oregon Green also were significantly elevated in cells treated with H₂O₂, which is consistent with little change in cytosolic pH caused by H₂O₂. In order to further confirm this, cytosolic pH in HL-60 cells with or without H₂O₂ pretreatment was experimentally determined and the pH was shown not to change to significant enough extent to alter drug accumulation (i.e., observed pH values were 7.57 ± 0.02 and 7.50 ± 0.03 , for cells with and without H₂O₂ treatment, respectively, $n = 3$, P -value < 0.04). Together, these findings strongly reduce the possibility that changes in intracellular pH could account for the observed changes in drug accumulation illustrated in Figure 3.

A second possibility for increased accumulation of these agents following H₂O₂ pretreatment could occur if the physical integrity of the plasma membrane was compromised as a result of the treatment. In other words, it might be

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possible that the H_2O_2 treatment could increase the “leakiness” of the plasma membrane which could facilitate the diffusion of drugs into the cytosol. To investigate this possibility we incubated cells with or without H_2O_2 pretreatment with Lucifer Yellow. This fluorescent dye is well-known to be membrane impermeable due to its polar nature (see Figure 1), and if the plasma membrane integrity was compromised we would expect to see increases in cytosolic accumulation of this molecule following H_2O_2 pretreatment. As is shown in Figure 4 there were no significant increases in cytosolic levels following the H_2O_2 treatment suggesting that the plasma membrane remained intact. Lucifer Yellow, as with any cell membrane impermeable molecule, typically enters cells only by fluid phase endocytosis. This is visually observed as punctate staining occurring in both cells with or without treatment, which can be observed in Figure 4.

A third possible explanation for enhanced drug accumulation following H_2O_2 pretreatment could be expected to occur if the agent caused changes in the fluidity of the plasma membrane. To evaluate this we utilized an increasingly popular technique for this purpose referred to as fluorescence recovery after photobleaching (FRAP).¹⁷ Briefly, a fluorescent molecule which incorporates specifically into the plasma membrane is incubated with cells. Then a strong fluorescent beam is focused on a small defined area of the plasma membrane to bleach the molecules in the beam's path. The rate at which unbleached molecules from surrounding portions of the plasma membrane diffuse to the bleached area is an indicator of plasma membrane fluidity. When comparing the recovery rate following bleaching we routinely observed that untreated cells were able to recover faster than cells treated with $50\ \mu\text{M}$ H_2O_2 (Figure 5). This result suggests that upon exposure to H_2O_2 the plasma membrane becomes more rigid. This is consistent with the work of others who have shown that oxidative stress typically leads to decreases in membrane fluidity.^{18,19} Intuitively, it would seem logical that increases in membrane fluidity should favor passive permeability; however, this is not the case. This phenomenon is actually consistent with the recent work of Sharma and colleagues who have evaluated a number of different intestinal absorption enhancers and found a correlation between membrane rigidity levels and increased passive permeability of a number of drugs.²⁰

It is important to point out that we do not believe this effect is caused by a H_2O_2 mediated reduction in the activity of drug transporter proteins that are often present on the

plasma membrane of multidrug-resistant cancer cell lines. Although we have not evaluated the fibroblast for expression of transporters, we have previously evaluated the HL-60 cell line and did not identify any transporters associated with this cell line using commercially available antibodies to common transporter proteins.¹⁴

The demonstrated correlation between H_2O_2 exposure and the concomitant increase in drug accumulation represents a substantial finding. Interestingly, additional studies (see Supplementary Figure 2) investigating the time and concentration dependence suggest that higher concentrations of H_2O_2 do not lead to more pronounced accumulation as may be expected. The results may suggest that patients experiencing oxidative stress (or an increase in H_2O_2 levels) may have an increased response to a given dosage of a drug relative to a patient with decreased oxidative stress, or those patients chronically taking antioxidants with their medications. This could be a very important factor in our continued efforts to provide more individualized dosing of drugs. Moreover, localized areas of the body receiving enhanced oxidative stress, such as the case with ischemia and reperfusion, may receive higher amounts of drugs relative to other areas of the body.

Conclusion

We believe that in the future it may be warranted to consider a patient's level of oxidative stress and/or H_2O_2 levels prior to making appropriate dosage recommendations for drugs with very narrow therapeutic indexes. Moreover, this work should significantly aid in the long-term goal of gaining a complete understanding, on a mechanistic level, of the myriad reasons responsible for variations in dose–response relationships among patients.

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Supporting Information Available: Figures depicting the influence of H_2O_2 exposure on the viability of the human leukemic cell line HL-60 and human skin fibroblasts using the Trypan Blue exclusion assay (Supplemental Figure 1) and results of investigation of drug accumulation in cells experiencing hydrogen peroxide pretreatment at variable hydrogen peroxide concentrations and exposure times (Supplemental Figure 2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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